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ORIGINAL RESEARCH

Characterization of Mucosal Immune-Related lncRNAs and mRNAs in a Mouse Model of Allergic Conjunctivitis

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Background: Allergic conjunctivitis (AC) is a common inflammatory condition characterized by immune dysregulation in response to environmental allergens. Despite extensive research into general allergic mechanisms, the specific immunological features of the ocular mucosal microenvironment remain poorly understood. Investigating immune-related mRNAs and LncRNAs may provide insights into the mechanisms underlying AC and potential novel targets for therapeutic intervention.

Methods: An AC model was established using female BALB/c mice sensitized with ragweed pollen. Conjunctival tissues from AC and control groups were pooled for RNA extraction, followed by Illumina sequencing. Differential gene expression was identified using DESeq2, and functional enrichment was analyzed using GO, KEGG, and GSEA. RT-qPCR validated results, while the Human Protein Atlas was used to assess protein expression.

Results: A murine model of AC was successfully established, confirmed by progressively increasing clinical scores and significantly elevated scratching frequency. Transcriptomic analysis revealed significant differences in mRNAs and lncRNAs expression between AC and control groups. GO analysis indicated that both upregulated and downregulated genes were enriched in biological processes related to response to stimulus, immune system processes, signaling, and metabolic processes. KEGG analysis showed that upregulated genes were enriched in pathways such as steroid hormone biosynthesis, histidine metabolism, glycolysis/gluconeogenesis, and IL-17 signaling, while downregulated genes were involved in cytokine-cytokine receptor interaction and hematopoietic cell lineage. GSEA identified significant enrichment in inflammatory pathways, including MAPK, STAT1, and STAT2. Mucosal immunity-related genes such as Bpifa1, Lcn2, and Reg3g were upregulated in AC. Co-expression analysis also revealed several upregulated lncRNAs, including Stoml3-202 and Etohd2-205.

Conclusion: This study is the first to systematically analyze immune-related mRNAs and LncRNAs in AC, identifying mucosal immunity molecules like Bpifa1 and Reg3g. These findings underscore the unique involvement of mucosal immunity in AC and provide potential new targets for immune modulation in ocular allergy treatment.

Keywords: allergic conjunctivitis, lncRNA, transcriptomic analysis, immune-related genes

Introduction

Allergic conjunctivitis (AC) is a common yet challenging inflammatory condition to treat effectively. Triggered by allergens such as pollen, animal dander, and environmental irritants, it presents with symptoms including red and itchy eyes, burning sensations, stinging, swelling, and tearing.[1] Despite its high prevalence, affecting 10–20% of the global population, the underlying mechanisms are not fully understood.[2,3] The widely accepted mechanism is that the immunopathogenesis of AC begins with the conjunctiva's initial exposure to environmental allergens.[4] These allergens disrupt the conjunctival epithelial barrier, allowing allergens to be captured by antigen-presenting cells (APCs) in the conjunctival stroma.[5] This results in the production of IL-4-secreting Th2 cells, which induce B cells to differentiate into plasma cells that secrete allergen-specific immunoglobulin E (IgE). IgE binds to FcεRI receptors on mast cells. Upon re-

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exposure to allergens, activated conjunctival mast cells degranulate, releasing mediators like histamine, lipid mediators, cytokines and chemokines, leading to the clinical manifestations of AC.[6]

The ocular surface is highly exposed to environmental threats and combats pathogenic threats. However, excessive inflammation can lead to ocular dysfunction, and even impair vision, potentially resulting in blindness.[7,8] To protect the eye, the ocular mucosa maintains immune tolerance through various mechanisms, exhibiting a lower immune response to external stimuli and thereby reducing the occurrence of excessive inflammatory reactions. This is distinct from the immune privilege observed within intraocular structures.[9,10] Peripheral immune tolerance involves various immunosuppressive factors and immune cells that create an anti-inflammatory microenvironment.[11] Currently, conjunctival goblet cells are known to secrete the immunosuppressive factor TGF-β.[12] Further research is needed to identify other secretions and cell types involved in ocular immune tolerance.

However, low immune response increases susceptibility to infections. To combat microbial infections, corneal and conjunctival epithelial cells respond to pathogens via functional Toll-like and NOD-like receptor signaling systems, secreting antimicrobial components and immunomodulatory factors like lysozyme, lactoferrin, and secretory antibody.[12–15] These mechanisms protect the eye, minimizing tissue damage and scar formation caused by inflammation. Due to these unique low immune response mechanisms, the pathogenesis of AC in the conjunctiva may differ from other tissues. For example, studies have shown that mast cells may not play a significant role in the development of ragweed-induced AC in mouse models.[16] Current research on AC often relies on classical immune mechanisms, lacking studies that focus on the unique immunological aspects of AC. This gap may impact our understanding of the allergic mechanisms occurring in the conjunctiva.

Non-coding RNA species, including long non-coding RNAs (LncRNAs), are integral in regulating immune responses, particularly in allergic diseases such as asthma, atopic dermatitis, and food allergies.[17] Recent studies have emphasized the involvement of LncRNAs in type-2 immune responses, which play a pivotal role in mediating allergic inflammation. Transcriptome sequencing (RNA-seq) is a powerful tool for profiling gene expression and discovering novel RNA molecules, including LncRNAs, that may be involved in the pathogenesis of these diseases.[18] However, obtaining conjunctival tissue samples from patients during allergic reactions remains challenging, resulting in a limited number of transcriptomic studies on AC.

In our study, we simulated clinically common seasonal AC by sensitizing mice with ragweed pollen to establish an AC model. We performed gene sequencing on the conjunctival tissues of these mice to identify immune-related mRNAs and LncRNAs. This research aims to elucidate the molecular mechanisms underlying AC and potentially provide new perspectives and therapeutic targets for future studies and treatment development.

Materials and Methods

Establishment of AC Model

All experimental procedures were approved by the Animal Ethics Committee of Jilin University (Approval No. 197) and strictly complied with China's national standard GB/T 35892–2018 "Guidelines for Ethical Review of Laboratory Animal Welfare". The study was conducted at the Laboratory Animal Center of Basic Medical Sciences, Jilin University, which holds the institutional animal care license (SYXK (Ji) 2023–0010) for SPF-grade rodents under barrier conditions. AC model was established as described previously.[19] Female BALB/c mice (8–10 weeks old) were purchased from Yisi Laboratory Animal Technology Co., Ltd. (Changchun, China). All animals were housed under standard laboratory conditions, including a 12-hour light/dark cycle, temperature of 22 ± 2°C, and humidity of 55 ± 5%, with food and water available ad libitum. Female BALB/c mice were chosen for their strong Th2 immune response, relevant to allergic reactions. On day 0, mice were sensitized via footpad injection of 100 μg short ragweed (SRW) pollen (Greer Lab, Lenoir, NC, USA) mixed with 2 mg Imject Alum (InvivoGen, San Diego, CA, USA), while control mice received Imject Alum alone. Starting on day 10, 10 μL of 50 mg/mL SRW pollen solution was administered to each eye daily for five consecutive days; control mice received 10 μL phosphate buffered solution (PBS), which was used as the diluent for SRW.

Clinical symptoms, including chemosis, conjunctival redness, eyelid edema, and tearing, were scored 20 minutes after each SRW challenge on a scale of 0 (absent) to 3 (severe). In addition, scratching behavior was recorded as a measure of ocular itch. The frequency of scratching was assessed during the challenge phase (Days 10 to 14), within 20 minutes after each SRW

administration. Scratching behavior was manually observed using a stopwatch in a double-blinded manner. A single scratching event was defined as a continuous contact of the eye area with either the forepaw or hind paw lasting ≥1 second, with at least a 2-second interval between events to avoid counting unconscious movements.

RNA Isolation and Sequencing

Conjunctival tissues from both eyes of two mice were pooled to generate a single RNA sample, yielding a total of 5 samples from 10 control mice and 5 samples from 10 AC model mice. This pooling was necessary due to the limited tissue volume from individual conjunctival samples, which restricts RNA yield. Total RNA was extracted from these pooled samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. RNA integrity was evaluated via standard denaturing agarose gel electrophoresis. RNA concentration for each sample was measured using a using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Agilent 2100 bioanalyzer (Agilent Technologies, SantaClara, CA, USA) was used to test the RNA integrity. Each RNA sample was then divided into two portions: one for transcriptome sequencing and the other for reverse transcription quantitative polymerase chain reaction (RT-qPCR).

RNA library preparation and RNA library sequencing were performed by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China). Prior to the construction of the RNA libraries, the rRNA Ribo-off rRNA Depletion Kit (Vazyme, N406, Nanjing, China) was used to remove the rRNA. Then, rRNA-depleted RNA was used to construct the RNA library with a VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (Vazyme, NR604, Nanjing, China). In the process, the extracted mRNA is enriched using mRNA Capture Beads. After purification with beads, the mRNA is fragmented using high temperatures. The fragmented mRNA is then used as a template to synthesize the first strand of cDNA in a reverse transcription enzyme mixture system. While synthesizing the second strand of cDNA, end repair and A-tailing are completed. Next, adapters are ligated, and VAHTS DNA Selection Beads are used for purification to select target fragments. PCR library amplification is then performed. The library quality was evaluated with a DNA 1000 assay Kit (Agilent Technologies, SantaClara, CA, USA). Finally, detection is carried out using the Illumina Novaseq X Plus by Gene Denovo Biotechnology Co. Ltd. (Guangzhou, China). All raw transcriptome data are accessible through Genome Sequence Archive (GSA) database.

To analyze lncRNAs and mRNAs, we first processed the sequenced reads using fastp (version 0.18.0). This step involved removing low-quality reads to ensure the retention of high-quality data. Subsequently, Clean reads were then compared to the ribosome database using Bowtie2 (version 2.2.8), retaining unmapped reads for later transcriptome analysis. Additionally, paired-end clean reads were matched to the mouse genome version release 112 from the Ensembl database with HISAT2 software[20] (version 2.1.0), and each measured lncRNA was annotated according to the corresponding genome annotation file. Transcriptome data are accessible through Genome Sequence Archive (GSA) database (accession numbers CRA020805).

RNA Sequencing Data Analysis

Differentially expressed genes between the control groups and the AC model groups were identified using differential expression analysis for sequence count data 2 (DEseq2) software. Genes were screened based on fold change and significance testing results. The criteria for selection included a fold change ≥ 1.5 and a p-value < 0.05. Partial bioinformatic analysis was performed using Omicsmart, a real-time interactive online platform for data analysis (http://www.omicsmart.com).

Functional Enrichment Analysis

To investigate the mechanisms underlying the differentially expressed lncRNAs and mRNAs in AC, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. These analyses aimed to predict potential signal transduction pathways, biological functions, and biochemical metabolic pathways associated with the differentially expressed RNA. The GO analysis encompasses three ontologies: biological process (BP), cellular component (CC), and molecular function (MF). This framework allows for the annotation of differentially expressed RNAs and facilitates the assessment of their functional significance. A hypergeometric test with a corrected p-value of < 0.05 was considered to

indicate significantly enriched GO terms and KEGG pathways. Gene Ontology (http://www.geneontology.org/), the KEGG database (http://www.genome.jp/kegg).

Gene Set Enrichment Analysis (GSEA) is a method used to analyze gene expression data through a gene set-based approach. To investigate the differences in biological processes between the control groups and the AC model groups, Gene Set Enrichment Analysis was performed utilizing the GSEA database (http://www.gsea-msigdb.org).

The PPI network of differentially expressed mRNAs was constructed using the STRING database (https://string-db.org). Interactions with a validation score greater than 0.4 were considered significant. The resulting networks were visualized using Cytoscape software (version 3.7.2).

Construction of lncRNA-mRNA Network

To investigate the latent functions of differentially expressed lncRNAs and their interactions with mRNAs, we constructed a co-expression network of lncRNA–mRNA transcripts. We calculated the Pearson correlation coefficient (PCC) to evaluate the correlation between these lncRNAs and mRNAs. The resulting co-expression network, which highlights these significant correlation pairs, was visualized using Cytoscape software (version 3.7.2).

Protein Expression in the Human Protein Atlas

Protein expression data for genes were obtained from the Human Protein Atlas (HPA, https://www.proteinatlas.org). The Institutional Review Board of China-Japan Union Hospital of Jilin University (IRB Exemption No. 2025-KYLL-003) confirmed this study's exemption from ethical review, as it involved only secondary analysis of anonymized data per China's ethical guidelines and institutional policies. Immunohistochemical (IHC) data were used to determine the localization of these proteins in tissues such as the lung, small intestine, cervix, and eye under normal physiological conditions. IHC protocols in the HPA involve antibody-based staining of tissue microarrays, ensuring high reproducibility. Additionally, single-cell RNA sequencing (scRNA-seq) data from the HPA were analyzed to assess gene expression in specific cell types, focusing on mucosal surfaces, particularly the lung and eye. This integrated approach highlighted the role of these molecules in mucosal immunity.

RT-qPCR

The SYBR green RT-qPCR assay was used to confirm the differentially expressed lncRNAs and mRNAs identified by transcriptome sequencing. Total RNA isolated from the tissue samples described above was used for reverse transcription and PCR. RT-qPCR was performed using the SYBR green assay kit (TaKaRa Biotechnology, Dalian, China) and a QuantStudio 3 Real-Time PCR System (Thermo Fisher, Waltham, MA, United States). The specific primers used for RT-qPCR are listed in Supplementary Table S1. The RT-qPCR program was as follows: denaturation at 95◦C for 30s, followed by 40 cycles at 95◦C for 5s, and 60◦C for 34s. Each sample was run in triplicate. GAPDH was the internal reference of lncRNA and mRNA. The relative quantitative analysis of the data was performed by $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

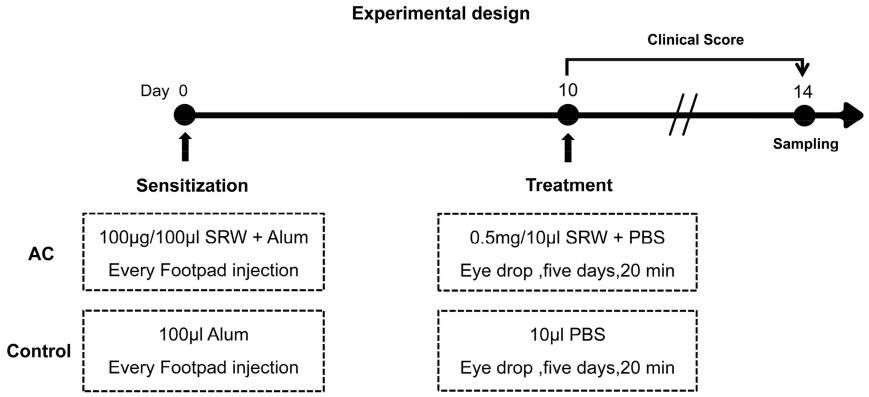
Data were expressed as the mean ± standard deviation. The fold change value and P-value were used to evaluate the differentially expressed LncRNAs and mRNAs. The significance of the difference of the expression levels of LncRNAs and mRNAs between the Control and AC groups was estimated using the Student's *t*-test. The false discovery rate (FDR) was calculated to correct the P-value. $P<0.05$ was regarded as statistically significant.

Results

Establishment and Evaluation of AC in a Murine Model

To establish and evaluate a murine model of AC, we used the method illustrated in Figure 1A. Mice were sensitized on day 0 with 100 μg/100 μL of SRW and alum via footpad injection. Starting on day 10, mice received daily eye drops of 0.5 mg/10 μL SRW and PBS for five days. As shown in Figure 1B, compared to the normal group, the AC model group exhibited significant eyelid edema (white arrows) and mucopurulent discharge (red arrows) from day 10 to day 14. Clinical symptoms were quantified using the scoring criteria in Figure 1C, which includes chemosis, conjunctival

A



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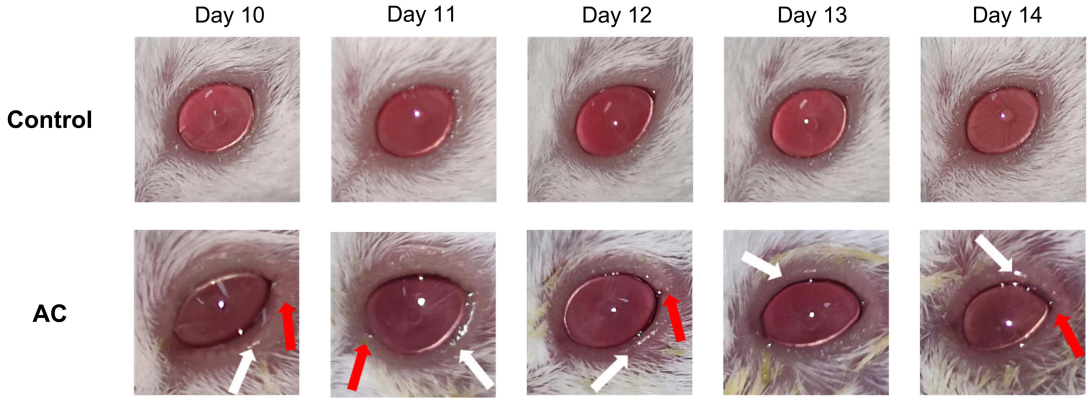
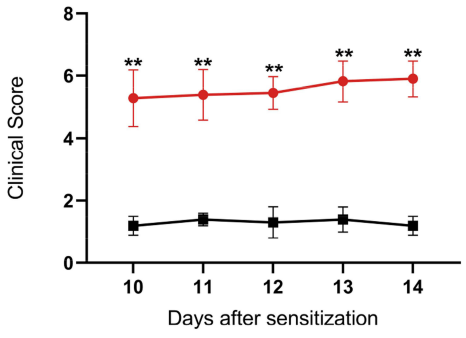


C

Clinical Evaluation of Allergic Conjunctivitis

	None 0	Mild 1+	Moderate 2+	Severe 3+
Chemosis	Absent	Mild chemosis	Obvious chemosis	Severe chemosis (lid margin 3-4 × thicker than baseline)
Conjunctival redness	Absent	Minimal redness	Moderate redness	Severe redness
Lid edema	Absent	Mild edema	Moderate edema	Severe edema (unable to open the eye)
Tearing and discharge	Absent	Mild	Moderate	Severe

D



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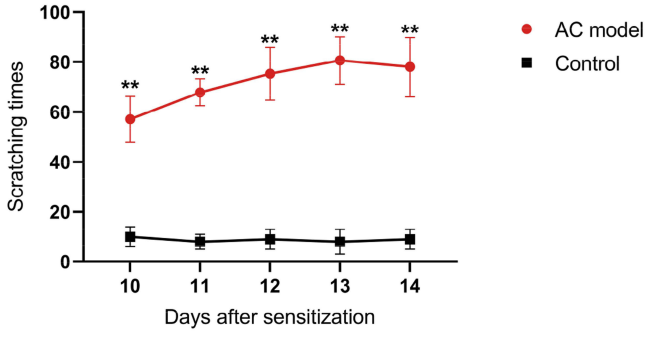

Figure 1 Experimental design and clinical evaluation of allergic conjunctivitis (AC) model. (**A**) Schematic illustration of the AC model. Mice were sensitized on day 0 with 100 μg/100 μL SRW mixed with alum via footpad injection. From day 11, mice received daily eye drops of 0.5 mg/10 μL SRW in PBS for five days. (**B**) Representative images of eye symptoms in the AC model from day 10 to day 14, showing eyelid edema (white arrows) and mucopurulent discharge (red arrows). (**C**) Clinical scoring criteria for AC severity. (**D**) Clinical score progression in the AC model group, showing an increase in severity from day 10 to day 14. (**E**) Scratching frequency, reflecting ocular irritation, is significantly higher in the AC model group than in controls. All the data (mean ± SD) were obtained from 10 mice per group. Statistical significance was calculated by Student's *t* test (**P<0.01).

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redness, lid edema, and tearing/discharge. Figure 1D shows a progressive increase in clinical scores from day 10 to day 14 in the AC model group. Additionally, the scratching frequency, a behavioral indicator of ocular irritation, was significantly higher in the AC model group compared to controls (Figure 1E). The above results confirmed the successful establishment of the AC model.

Comparative Analysis of mRNAs and lncRNAs Characteristics in AC

The distribution of exon numbers between lncRNAs and mRNAs shows a significant difference. lncRNAs predominantly have fewer exons compared to mRNAs, while mRNAs display a broader distribution across multiple exon numbers (Figure 2A). The length of lncRNA transcripts is generally shorter compared to mRNAs (Figure 2B). Various lncRNA types are classified into several categories, including sense, antisense, intronic, bidirectional, intergenic, and others LncRNA. The majority of lncRNAs fall into the sense LncRNA category (Figure 2C). Heatmaps exhibit distinct differential expression profiles of mRNAs and lncRNAs between control and AC samples (Figure 2D). The volcano plots, generated by screening with log2 fold change (FC)>1 and p-value<0.05, highlight significantly upregulated and downregulated genes in AC samples (Figure 2E).

Functional Enrichment Analysis of Differentially Expressed Genes

To investigate the biological functions of the differentially expressed genes between health and AC, GO enrichment analysis was performed. As shown in Figure 3A, upregulated genes in terms of biological process are involved in response to stimulus, metabolic processes, signaling, developmental processes, and immune system processes. For molecular function, binding is prominently represented, with enrichment also seen in catalytic activity and molecular transducer activity. In terms of cellular component, cellular anatomical entities and protein-containing complexes are enriched. As shown in Figure 3B, downregulated genes in terms of biological process are primarily associated with response to stimulus, metabolic process, signaling, positive regulation of biological process and immune system process. For molecular function, binding is the most significantly enriched category. In terms of cellular component, cellular anatomical entities and protein-containing complexes are also enriched.

KEGG pathway analysis was employed to understand the pathways in which the different expressed genes are involved. As shown in Figure 3C, the upregulated genes show significant enrichment in pathways such as steroid hormone biosynthesis (ko00140), histidine metabolism (ko00340), glycolysis/gluconeogenesis (ko00010), and IL-17 signaling pathway (ko04657), which is associated with inflammation. For the downregulated genes, KEGG pathway analysis indicates significant involvement in pathways associated with cytokine-cytokine receptor interaction (ko04060), hematopoietic cell lineage (ko04640), and inflammatory bowel disease (ko05321), as shown in Figure 3D.

Functional and Pathway Enrichment Analysis of Differentially Expressed lncRNAs in AC

To elucidate the biological functions of differentially expressed lncRNAs in AC, GO enrichment analysis and KEGG pathway analysis were performed. GO enrichment analysis of upregulated lncRNAs revealed significant enrichment in various biological processes, molecular functions, and cellular components. Upregulated lncRNAs are notably involved in response to stimulus, multicellular organismal process, signaling and metabolic process. Binding is prominently represented among molecular functions, and cellular anatomical entity are significantly enriched within cellular components (Figure 4A). Downregulated lncRNAs are primarily associated with response to stimulus, metabolic process, multicellular organismal process and signaling. Molecular functions such as binding and catalytic activity are notably enriched, as well as cellular components including cellular anatomical entity and protein-containing complex (Figure 4B).

KEGG pathway analysis for upregulated lncRNAs highlights significant enrichment in pathways related to pentose and glucuronate interconversions (ko00040), steroid hormone biosynthesis (ko00140), beta-Alanine metabolism (ko00410), and lysosome (ko04142) (Figure 4C). For downregulated lncRNAs, KEGG pathway analysis indicates significant involvement in pathways associated with cytokine-cytokine receptor interaction (ko04060), inflammatory bowel disease (ko05321), hematopoietic cell lineage (ko04640), and the IL-17 signaling pathway (ko04657) (Figure 4D).

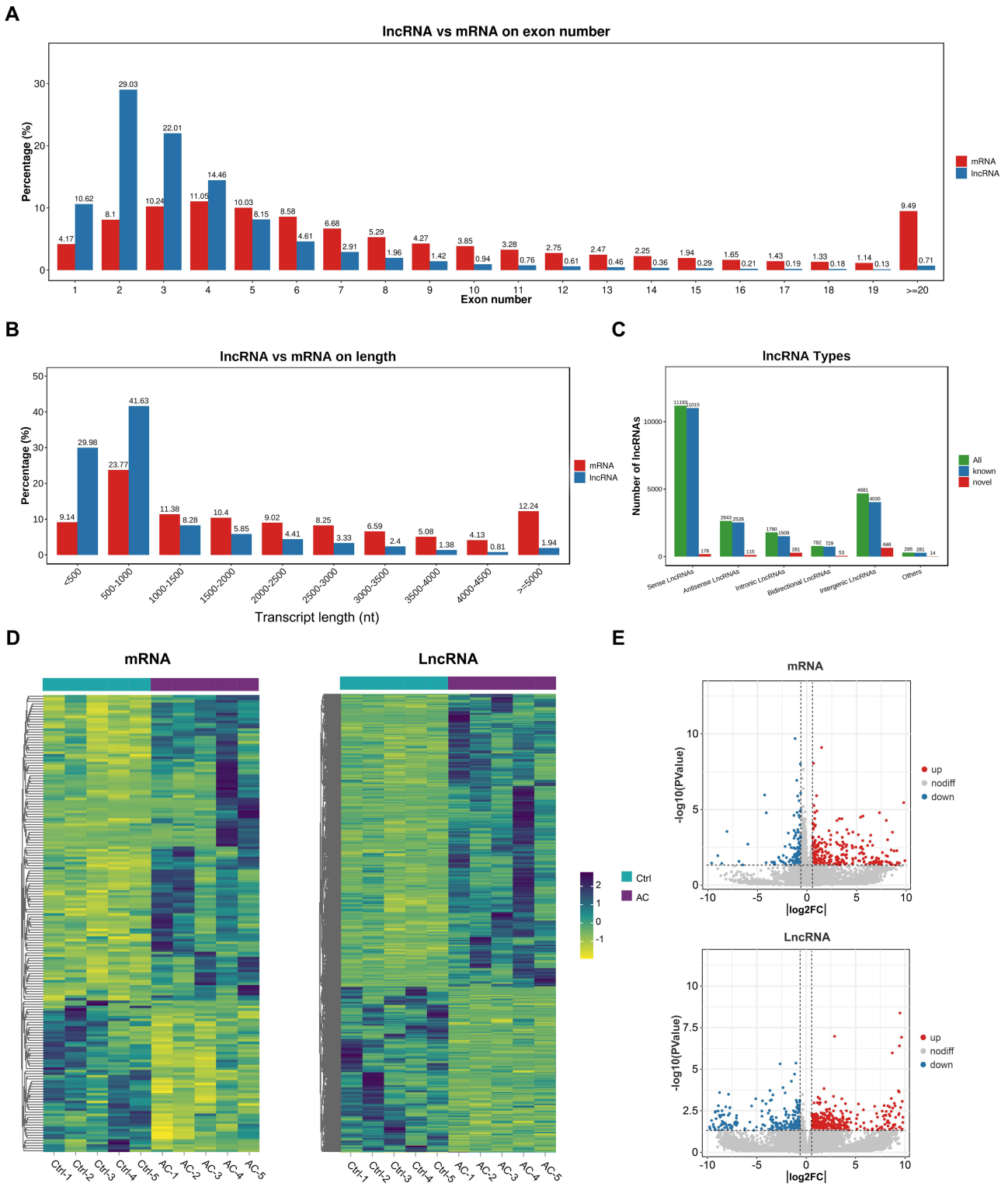

Figure 2 Comparative analysis of lncRNAs and mRNAs in AC. (**A**) Comparison of exon number distribution between lncRNAs and mRNAs. (**B**) Transcript length distribution of lncRNAs and mRNAs. (**C**) Classification of lncRNA types. (**D**) Heatmaps depicting distinct expression profiles of mRNAs and lncRNAs between control and AC samples. (**E**) Volcano plots of mRNA and lncRNA differential expression, highlighting significantly upregulated and downregulated genes (log2 FC > 1, p-value < 0.05).

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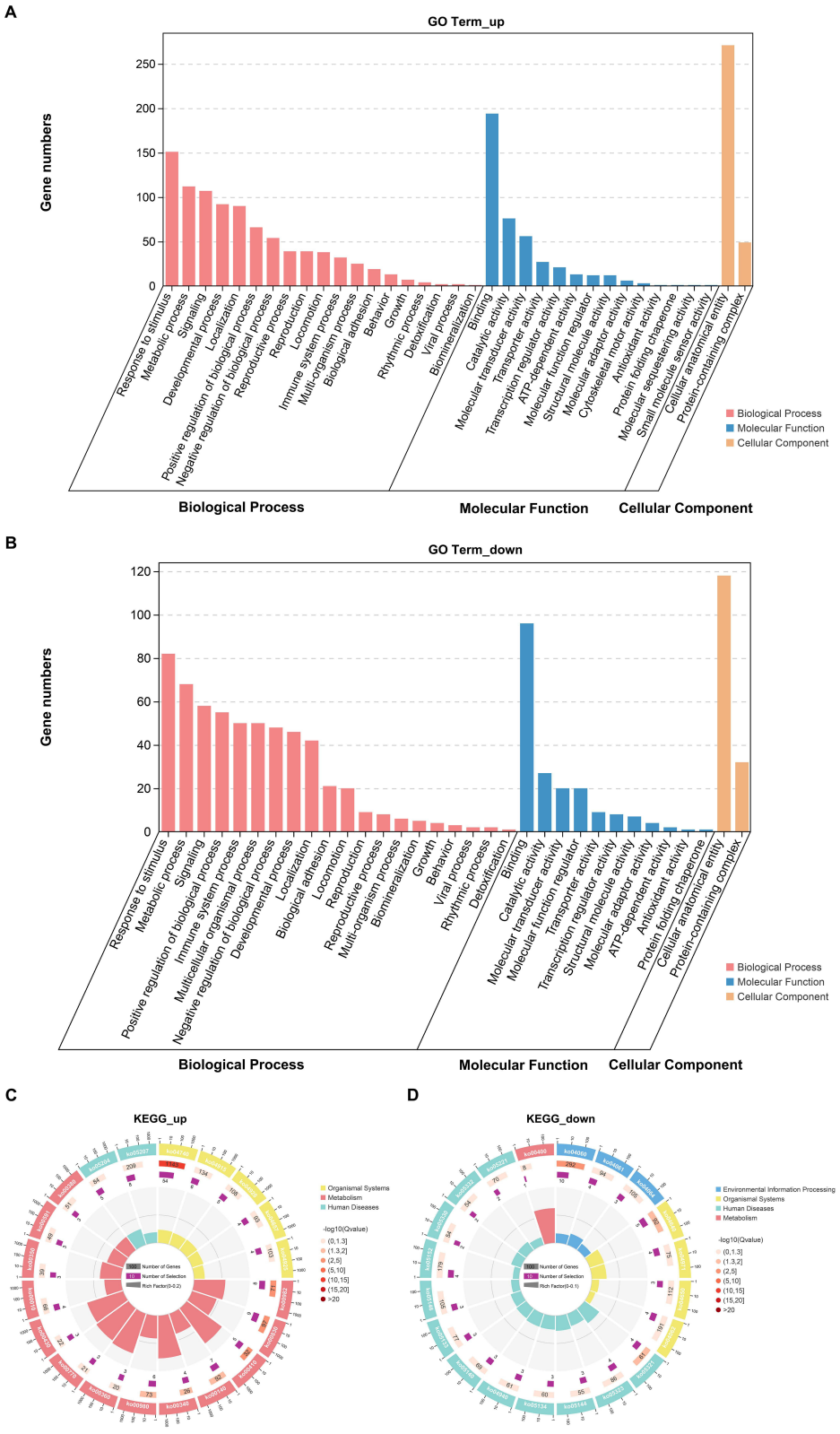

Figure 3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes in AC. GO enrichment analysis of upregulated genes (**A**) and downregulated genes (**B**), categorized by Biological Process, Molecular Function, and Cellular Component. KEGG pathway enrichment analysis of upregulated genes (**C**) and downregulated genes (**D**), showing significantly enriched pathways associated with AC.

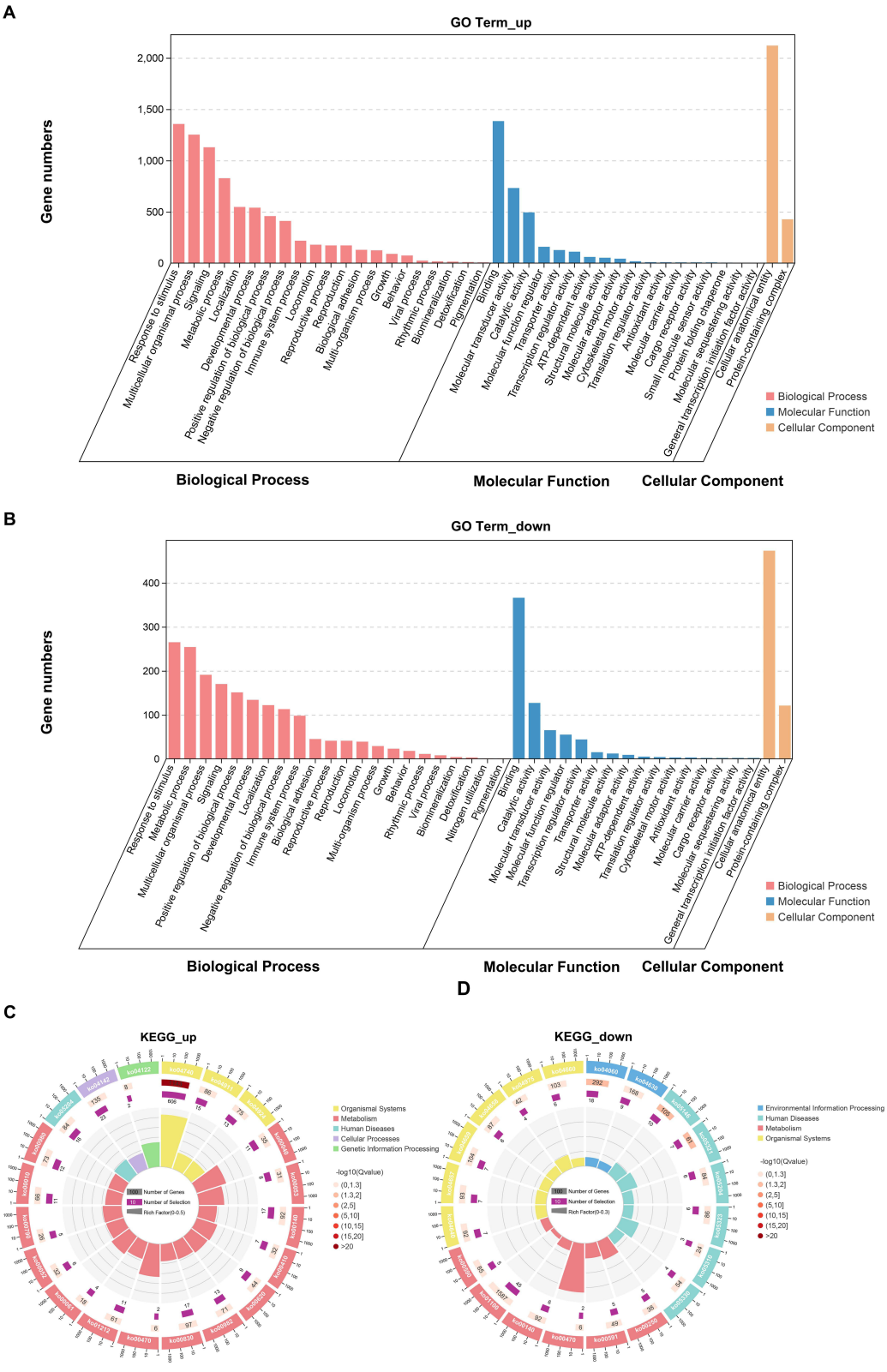

Figure 4 GO and KEGG enrichment analysis of differentially expressed LncRNA in AC. GO enrichment analysis displays upregulated LncRNAs (**A**) and downregulated LncRNAs (**B**), categorized into Biological Process, Molecular Function, and Cellular Component. KEGG pathway enrichment analysis shows upregulated LncRNAs (**C**) and downregulated LncRNAs (**D**), highlighting significantly enriched pathways associated with AC.

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Inflammation-Related Signaling Pathways and Key Immune Genes in AC

AC is characterized by significant involvement of the immune system in its pathophysiology. To further analyze the inflammation-related signaling pathways involved in AC, we utilized REACTOME for GSEA, which is effective in identifying pathways based on biological knowledge. As shown in Figure 5A, the GSEA results revealed significant enrichment in several pathways, including immune response in mucosa, xenobiotics metabolism, cell-cell communication, arachidonic acid metabolism, and multiple phosphorylation pathways such as mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 1 (STAT1), and signal transducer and activator of transcription 2 (STAT2). These changes indicate that AC is intricately linked to inflammatory and immune responses.

In Figure 5B, we aimed to identify key immune-related genes, which were obtained from the IMMPORT database. A Venn diagram showed the intersection of differentially expressed mRNAs and immune-related genes (IRGs), revealing 17 upregulated and 21 downregulated IRGs. Subsequently, Figure 5C highlights the top 20 significantly differentially expressed genes. GO analysis (Figure 5D) of the upregulated IRGs indicated involvement in processes like the innate immune system, Toll-like receptor signaling, and antimicrobial peptide activity. Co-expression analysis (Figure 5E) identified core genes, including Bpifa1, Lcn2, Reg3g, Bpifb3, Bpifb6, Bpifb4 and Pltp.

Mucosal Immunity-Related Genes in AC

Interestingly, the core genes identified are not typical allergy-associated molecules but are primarily involved in mucosal immunity, with direct antimicrobial functions. In Figure 6A, immunohistochemical analysis using the Human Protein Atlas database revealed that Bpifa1, Lcn2, and Reg3g, molecules with direct antimicrobial activity, are expressed under physiological conditions in the mucosa of the lung, small intestine, and cervix, but are not expressed in smooth muscle and the cerebral cortex. Since the conjunctiva is also a mucosal surface, we used single-cell analysis from the Human Protein Atlas to compare the expression of these genes in the lung and eye. As shown in Figure 6B, single-cell analysis indicates that in the lung, these molecules are primarily secreted by club cells and ciliated cells, both types of airway epithelial cells. However, under physiological conditions, Bpifa1, Lcn2, and Reg3g are not expressed by any cell types in the eye.

To explore the relationship between these antimicrobial molecules and LncRNAs, we conducted a co-expression analysis, as shown in Figure 6C. To assess the expression of these molecules in the conjunctiva in response to AC, we performed PCR analysis on conjunctival tissue from AC mice. The result showed that antimicrobial molecules such as Bpifa1, Bpifb3, Bpifb4, Bpifb6, and Reg3g are upregulated in the AC group compared to the control group (Figure 6D). PCR validation revealed that Stoml3-202, Etohd2-205, D3Ertd751e-209, Myrf-206, and Eps15l1-206 were significantly upregulated in AC (Figure 6E). This suggests that these LncRNAs may contribute to the ocular immune response in AC by regulating the expression of antimicrobial molecules.

Discussion

AC is a common clinical manifestation of allergy-related immune dysregulation.[21] While allergic mechanisms have been extensively studied, few investigations have addressed the unique immunological features of the ocular mucosal microenvironment, highlighting the need to better understand its specific molecular processes. In this study, we established a murine model of seasonal AC using ragweed pollen, which closely mimics the allergic responses observed in human AC.[22] This model allowed us to perform comprehensive transcriptomic analyses to explore the roles of mRNAs and LncRNAs in the pathogenesis of AC.[23] Our findings clarify the immune and inflammatory pathways in AC and identify key genes and LncRNAs associated with mucosal immunity that may regulate conjunctival immune responses.

The transcriptomic analysis revealed distinct differences in the expression of both mRNAs and LncRNAs between the control and AC groups. GO analysis indicated that both upregulated and downregulated genes in AC are involved in immune system processes. KEGG analysis showed that pathways such as steroid hormone biosynthesis, histidine metabolism, and the immune-related IL-17 signaling pathway were upregulated in AC. Among these, the Steroid hormone biosynthesis pathway may play a protective role in mucosal immunity during allergic reactions. Allergic responses can lead to the activation of local immune cells, which, in turn, increase cortisol synthesis.[24] Cortisol modulates immune responses through negative feedback, potentially alleviating excessive inflammation in the

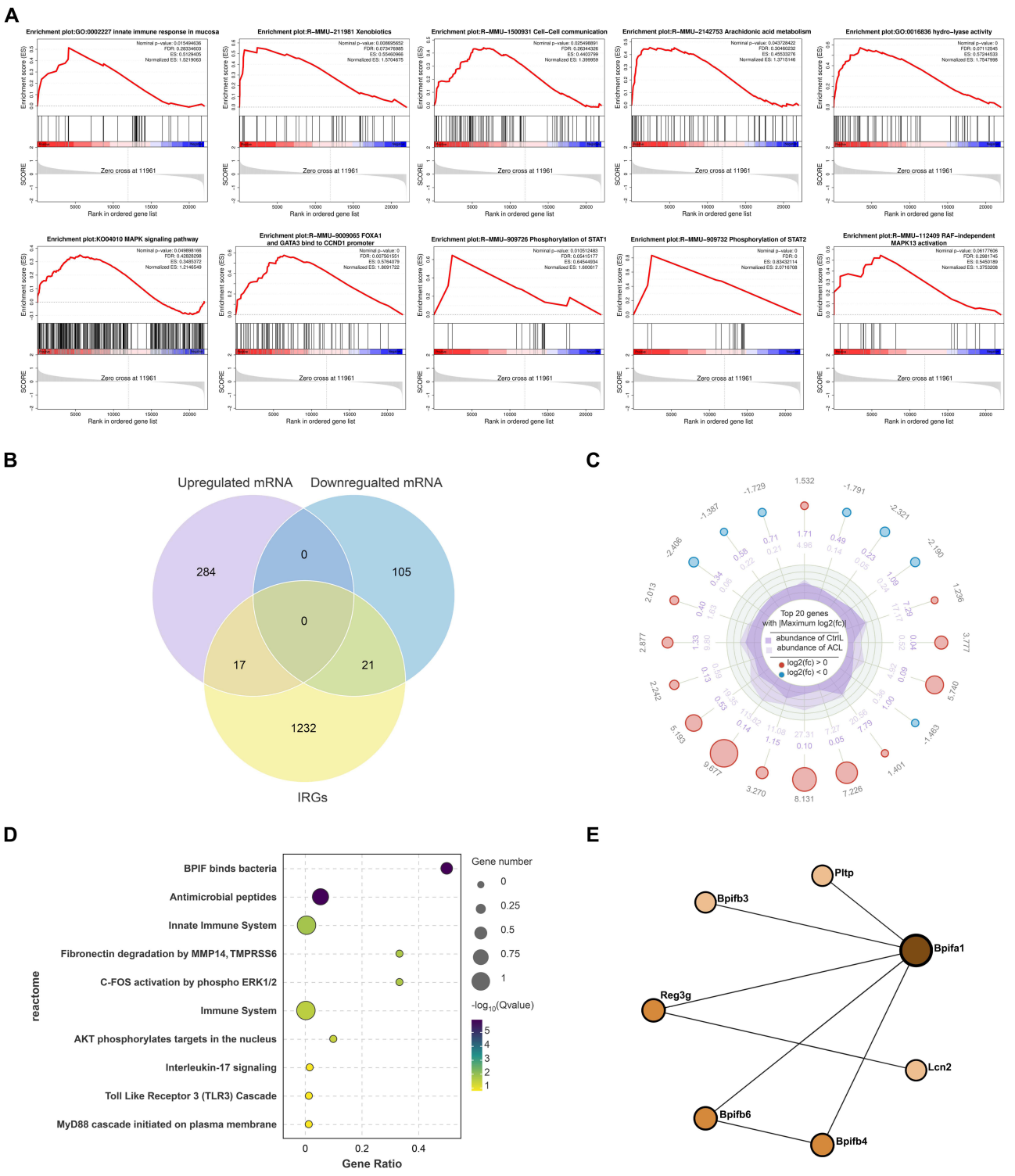

Figure 5 Analysis of differentially expressed immune-related genes (IRGs) in AC. (**A**) Gene Set Enrichment Analysis (GSEA) of differentially expressed genes in AC. Each plot shows the enrichment score (red line) across the ranked gene list, with peaks indicating significant enrichment in specific pathways. Positive and negative enrichment scores reflect upregulation or downregulation in AC, respectively. (**B**) Venn diagram illustrating the overlap between upregulated and downregulated mRNAs and IRGs. (**C**) Top 20 differentially expressed immune-related genes, displayed with a circular bar plot where the radius indicates gene expression log-fold changes, with red denoting upregulated and blue denoting downregulated genes. (**D**) GO enrichment analysis of upregulated IRGs. Dot size represents the number of genes in each pathway, while color intensity indicates statistical significance based on –log10(p-value). (**E**) Co-expression network of key IRGs. Node size indicates the correlation coefficient of co-expression. The depth of node color reflects the number of co-expressed genes, with darker brown indicating more connections and lighter brown indicating fewer.

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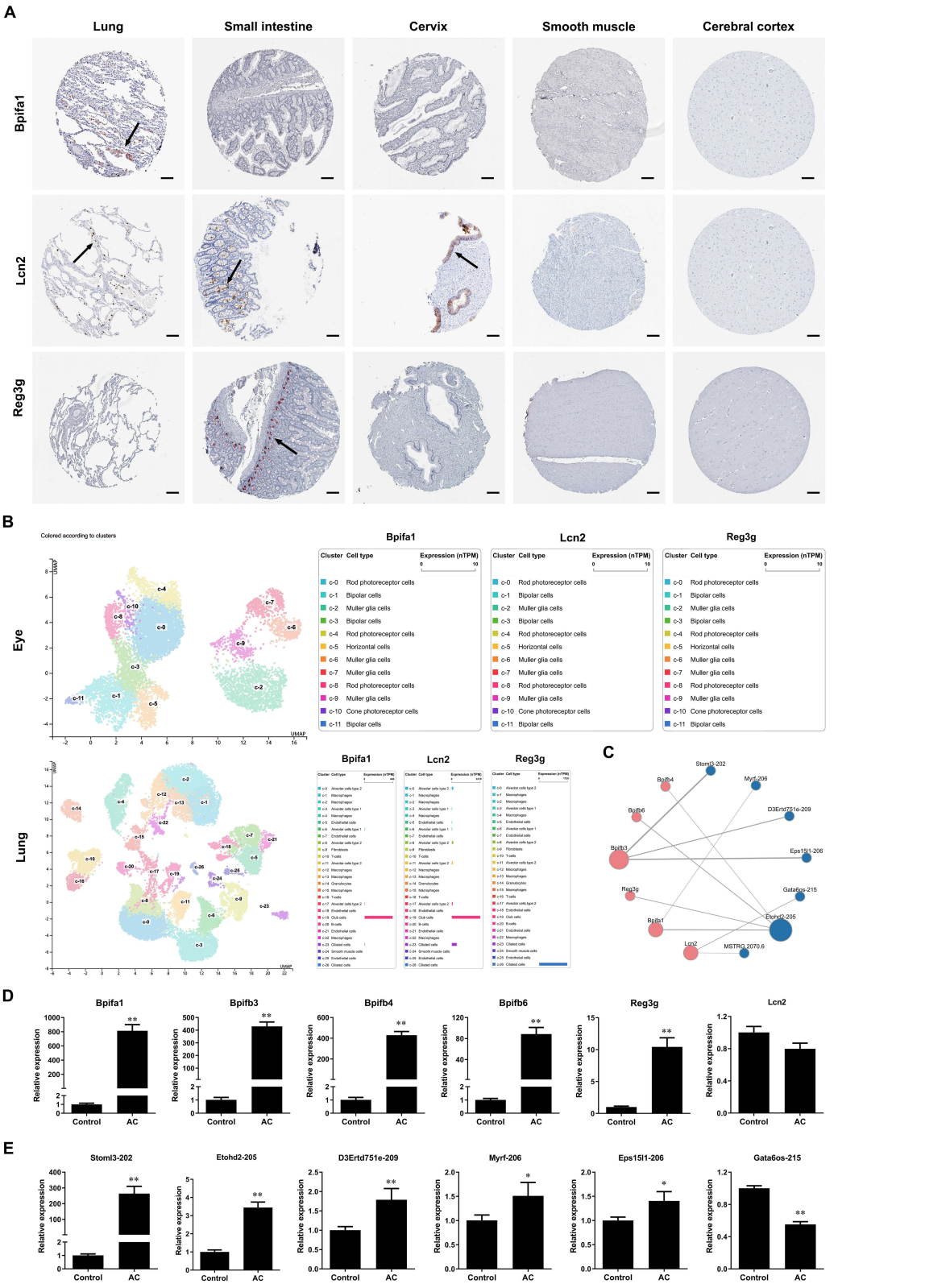

Figure 6 Expression of mucosal immunity-related genes and associated LncRNAs in AC. (**A**) Immunohistochemical analysis of Bpifa1, Lcn2, and Reg3g expression in various tissues (lung, small intestine, cervix, smooth muscle, and cerebral cortex) under physiological conditions, using data from the Human Protein Atlas (HPA) database. Arrows indicate regions of positive staining, with darker staining representing higher protein expression levels.Scale bar=100 μm. (**B**) Single-cell RNA sequencing analysis of Bpifa1, Lcn2, and Reg3g expression in lung and eye tissues, highlighting specific cell types and expression levels represented by colored bars; bar length indicates expression quantity. (**C**) Co-expression network of key immune-related genes and associated LncRNAs identified in AC. Red nodes represent mRNAs, while blue nodes represent LncRNAs; node size indicate co-expression strength. PCR validation of mRNA (**D**) and LncRNA (**E**) expression levels in control and AC mouse conjunctival tissues. All the data (mean ± SD) were obtained from 5 mice per group. Statistical significance was calculated by Student's *t* test (*P<0.05, **P<0.01).

conjunctiva.[25] This could act as a protective mechanism against ocular tissue damage.[26] Another key pathway identified is histidine metabolism. Histidine is a precursor of histamine, a critical bioactive molecule mediating allergic responses.[27,28] Thus, increased histidine metabolism following AC induction may intensify allergic symptoms by enhancing histamine production.

In addition, we found it intriguing that the IL-17 signaling pathway was upregulated following AC. IL-17 is typically elevated in bacterial infections, as it plays a critical role in activating neutrophils, enhancing barrier functions, and promoting antimicrobial immune responses.[29–31] However, recent studies have indicated that IL-17 levels also tend to rise in certain allergic conditions, such as asthma and atopic dermatitis, suggesting its potential involvement in allergic responses.[32–34] This upregulation of IL-17 may indicate an attempt by the immune system to bolster local barrier defenses in response to compromised mucosal integrity in allergic patients, potentially reducing susceptibility to secondary infections.

In addition to the changes in mRNA expression, we also identified a large number of differentially expressed LncRNAs. LncRNAs play a crucial role in regulating gene expression at both transcriptional and post-transcriptional levels. The results of the GO analysis indicated that these differentially expressed LncRNAs are involved in immune system-related biological processes, suggesting that they may play an important role in immune regulation in AC.

To further explore the potential functions of these LncRNAs, we conducted KEGG pathway analysis, which revealed that the differentially expressed LncRNAs are associated with steroid hormone biosynthesis and the IL-17 signaling pathway. Interestingly, these pathways overlap with those identified in the mRNA KEGG analysis, suggesting that both LncRNAs and mRNAs may play crucial roles in the development of allergic conjunctivitis (AC). This consistency implies that LncRNAs and mRNAs may be involved in the same or closely related physiological processes, where LncRNAs potentially regulate gene expression associated with mRNA, influencing the activity of key biological pathways and processes.

Given the pivotal roles of these pathways, it is likely that LncRNAs play a significant role in modulating immune responses and pathological processes in AC, possibly in collaboration with mRNAs. This insight into the molecular mechanisms underlying AC offers new potential therapeutic targets, particularly for LncRNAs that are closely related to immune and inflammatory pathways. Considering that current treatments for AC, such as corticosteroids and antihistamines, may have associated side effects, further exploration of LncRNA's role in immune regulation could lead to more precise and targeted therapies. By targeting LncRNAs, it may be possible to develop therapeutic strategies that reduce inflammation and improve immune modulation, offering a potentially safer and more effective approach to managing AC.

GSEA is a method used to identify significant enrichment of gene sets involved in specific biological functions. In the context of AC, we applied GSEA to explore immune-related pathways altered during the allergic response. Following AC induction, genes were significantly enriched in several immune-related pathways, including MAPK, STAT1, and STAT2 pathways.[35,36] The enrichment of these pathways suggests increased activation of inflammatory signaling and allergic responses. The MAPK pathway is known to play a crucial role in mediating inflammatory responses, while STAT1 and STAT2 are key players in cytokine signaling, potentially indicating enhanced immune activation and inflammation following allergen exposure in AC.[37–39] These findings imply that allergic inflammation in AC involves coordinated activation of multiple pro-inflammatory signaling networks.

Further analysis of immune-related differentially expressed genes in AC revealed an interesting pattern: several key upregulated genes, including Bpifa1, Lcn2, and Reg3g, are primarily recognized for their roles in mucosal immunity and antimicrobial defense, rather than for the classic pro-inflammatory functions typically associated with allergic responses. Bpifa1 is known for its broad expression in respiratory epithelium, where it contributes to innate immune defense against pathogens and helps maintain epithelial barrier integrity.[40,41] Lcn2, an iron-binding protein, limits bacterial growth and modulates immune responses, providing protective effects in various mucosal tissues.[42,43] Similarly, Reg3g, a C-type lectin, has notable antimicrobial activity, particularly in the gut, where it prevents bacterial invasion and supports mucosal homeostasis.[44,45] These findings suggest that mucosal immunity-related genes, traditionally associated with infection defense, may play a novel role in modulating the ocular immune response during AC.

Interestingly, prior research has not reported the expression of these mucosal immunity-related antimicrobial molecules in the context of AC. Our study demonstrates their upregulation in the conjunctiva during AC, suggesting that they may play a key role in modulating immune responses at the ocular surface. Given the constant exposure of the conjunctival epithelium to environmental pathogens and allergens, the upregulation of these immune defense molecules could represent a unique

mechanism by which the conjunctiva responds to allergic inflammation. This response may help prevent microbial invasion while simultaneously regulating excessive inflammatory responses, thus protecting the ocular tissue from damage.

However, it remains unclear whether these molecules are directly involved in regulating the inflammatory response in AC, in addition to their potential role in antimicrobial protection of the mucosal barrier. The exact mechanisms underlying the upregulation of these molecules during AC induction are still unknown. Therefore, further research is needed to elucidate the precise functions of these molecules in AC, as well as the mechanisms by which they modulate the severity of allergic inflammation in the conjunctiva.

Conclusions

In conclusion, this study identifies key mRNAs and LncRNAs that are differentially expressed in the conjunctiva of a murine model of AC. These molecules are associated with immune modulation and mucosal defense mechanisms, and may play important roles in regulating conjunctival immune responses. Notably, we found that several mucosal immunity-related molecules, such as Bpifa1 and Reg3g, were upregulated in AC, suggesting that they may contribute to the adaptive immune response at the ocular surface. Additionally, the involvement of LncRNAs in regulating immune-related genes highlights their potential role in immune responses in AC. However, several limitations exist in this study. First, validation in human conjunctival samples is necessary to assess the clinical relevance of these findings. Second, additional experimental studies are required to further elucidate the exact mechanisms by which these mucosal immunity-related genes and LncRNAs contribute to the pathogenesis of AC. Future research will be crucial to better understand their roles in ocular immune responses and their potential implications for the treatment of AC.

Data Sharing Statement

The original data presented in this study are publicly available and can be found at: https://ngdc.cncb.ac.cn/gsa/browse/CRA020805.

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Disclosure

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