The role of inflammasomes in the pathogenesis of nephrotic syndrome in children

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The role of inflammasomes in the pathogenesis of nephrotic syndrome in children

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ABSTRACT

The role of inflammasomes in the pathogenesis of nephrotic syndrome in children

INTRODUCTION: Idiopathic nephrotic syndrome (INS), particularly steroid-sensitive nephrotic syndrome (SSNS), is characterized by recurrent proteinuria, necessitating extended steroid therapy. This prolonged treatment often subjects patients to substantial side effects. Although the etiology of SSNS remains poorly understood, this study aimed to explore its pathogenesis with a focus on the role of the inflammasome.

METHODS: This study was the first to utilize blood from patients with SSNS in both acute and chronic remission phases of the disease and to stimulate podocytes *in vitro*, aiming to uncover mechanisms related to inflammasomes. The investigation included patients with a confirmed diagnosis of SSNS, aged 4 to 18 years, who had provided informed consent between 2016 and 2023. Subjects presenting active proteinuria were designated to the acute phase group, whereas those who had sustained a relapse-free state for more than one year without pharmacological intervention were allocated to the chronic remission phase group. During clinical assessments, venipuncture was performed to collect a 7.5 cc blood sample from each participant. Of this, 2.5 cc was stored in liquid nitrogen for subsequent messenger RNA (mRNA) sequencing. For the analysis, aliquots of whole blood from patients in both the acute and chronic remission phases were utilized, with data analyzed using various statistical models. The remaining 5 cc was immediately centrifuged, and the separated serum was cryopreserved in liquid nitrogen at −140 ℃ for future use in the *in vitro* culture of human podocytes. Cultured cells were segregated into four distinct groups for experimental manipulation: a control group receiving vehicle alone, a group stimulated with sera from the acute phase, a group treated with acute phase sera concomitantly with dexamethasone (DEX), and a group incubated with sera from the chronic remission phase. After treatment, these podocytes were evaluated for changes in intracytoplasmic actin filaments and subjected to mRNA sequencing. Data derived from these experimental conditions were analyzed using sophisticated statistical modeling to elucidate the molecular underpinnings of SSNS.

RESULTS: Analysis of whole blood from patients in the acute and chronic remission phases of SSNS revealed statistically significant differences in genes associated with inflammasomes, including Nod-like receptor (NLR) family caspase activation and recruitment domain containing 4 (*NLRC4*), nucleotide-binding domain and leucine-rich repeat-containing family apoptosis inhibitory protein (*NAIP*), nuclear factor-kappa B1 (*NFKB1*), and mitogen-activated protein kinase 14 (*MAPK14*). A total of 3,554 differentially expressed genes (DEGs) were identified between the two groups. Gene ontology (GO) enriched pathway analysis indicated that programmed cell death (PCD), apoptotic processes, cellular biosynthetic processes, and intracellular signal transduction involving the MAPK pathway were upregulated in the acute phase group.

Experiments stimulating podocytes with patient sera demonstrated a reduction in intracytoplasmic actin filaments when exposed to acute phase sera, which was reversed upon the addition of DEX, restoring the clarity of actin filament staining. Podocytes treated with acute phase sera and DEX showed significantly reduced expression of caspase 3 (*CASP3*), interleukin-1β (*IL1B*), and *NFKB1* compared to cells stimulated with acute phase sera alone. Among the 2,153 DEGs identified between the two groups, 79 were found to overlap with the PodNet database. GO enrichment analysis revealed that upregulated DEGs in acute phase sera stimulation were enriched for cytoskeleton organization and supramolecular fiber organization pathways, while downregulated DEGs were enriched in processes such as PCD, apoptotic processes, and metabolic processes.

CONCLUSION: This study suggests a significant association of NAIP/NLRC4 inflammasomes, which represent a different pathway from NLR family pyrin domain containing 3 (NLRP3), with the pathogenesis of SSNS. NAIP/NLRC4 inflammasomes and related pro-inflammatory cytokines may serve as a potential link between bacterial infections or abnormalities in the immune response and the onset or exacerbation of proteinuria. The imbalance between pro-inflammatory and anti-inflammatory signals can induce pathological changes in podocytes. Further research is needed to elucidate the specific roles and relationships of these substances in nephrotic syndrome and to develop additional therapeutic strategies.

Key words: Nephrotic syndrome, Idiopathic nephrotic syndrome (INS), Steroid-sensitive nephrotic syndrome (SSNS), Inflammasome, Human podocytes

I. INTRODUCTION

Idiopathic nephrotic syndrome (INS) is a recurrent kidney disease characterized by significant proteinuria, leading to edema and hyperlipidemia. Predominantly affecting pediatric populations, the most common type of INS is steroid-sensitive nephrotic syndrome (SSNS), which generally elicits a favorable response to corticosteroids. However, about 50% of patients may experience frequent relapses or become steroid-dependent for disease control,[1] often necessitating continued corticosteroid therapy until at least puberty. While long-term corticosteroid use can prolong remission, it also leads to various complications such as cushingoid features, behavioral changes, cataracts, hypertension, osteoporosis, and growth retardation.[2] Therefore, advancing the development of therapeutic drugs that could potentially serve as alternatives to steroids is crucial.

Current research suggests that SSNS could result from direct podocyte injury induced by certain circulating factors.[3-5] However, the exact etiology of SSNS remains unknown, and there is a shortage of studies clearly elucidating the causative factors and mechanisms, particularly in children with SSNS.

In 2002, Jürg Tschopp and others discovered a mechanism for cytokine interleukin (IL)-1 production, a potent inflammatory factor.[6] They identified a large protein complex that activates caspase-1, which is required to produce IL-1, and named it the inflammasome.[6] As research progressed, it was discovered that three Nod-like receptor (NLR) proteins, namely NLR family pyrin domain containing 1 (NLRP1), NLRP3, and NLR family caspase activation and recruitment domain (CARD) containing 4 (NLRC4), activate caspase-1 and facilitate the cleavage of precursors of IL-1 and IL-18 to produce mature inflammatory cytokines.[6] Among these, the NLRP3 inflammasome is the most studied and well-known.

NLRP3 is expressed in monocytes, macrophages, neutrophils, dendritic cells, some lymphocytes, and epithelial cells.[7] NLRP3 can be activated by a variety of components derived from bacteria, fungi, and some viruses.[8] In addition to microbial components, NLRP3 can be activated by hyaluronan and beta-amyloid (associated with Alzheimer's plaques), and extracellular adenosine triphosphate (ATP) or glucose, which are secreted by damaged tissues or cells.[7] Recent studies have shown that the NLRP3 inflammasome mediates severe inflammatory conditions caused by abnormal harmful substances, such as uric acid crystals.[7] It is also thought to be involved in the activation of various stimuli through a series of signals, such as the release of potassium ions, reactive oxygen

species (ROS), the activity of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, and the outflow of lysosomal components.[9] All these signals induce the assembly of the NLRP3 inflammasome, activation of caspase-1, and the subsequent cleavage and secretion of IL-1.[10] These findings indicate that mature IL-1 is synthesized in response to innate and inflammatory stimuli. However, questions remain regarding the mechanism by which the activated inflammasome exerts the function of caspase-1 and several other unknown NLR proteins.

Efforts have been made to investigate the connection between inflammasomes and renal diseases, with several studies focusing on diabetic nephropathy,[11] IgA nephropathy,[12,13] Lupus nephritis,[14] and more. Nevertheless, there is presently a limited amount of research distinctly clarifying the association between inflammasomes and nephrotic syndrome, especially in cases of SSNS. While there is a study that sheds light on this relationship through experiments in mice,[15] research utilizing human serum remains scarce.

In this study, our primary objective was to initiate *in vivo* experiments by first analyzing the transcriptomes of whole blood from SSNS patients to identify genes of interest. In addition, *in vitro* experiments were used to explore the relationship between podocytes and SSNS patient sera *via* co-culturing. We further determined whether the podocytes undergo reversal with steroids. Finally, we monitored alterations in intracytoplasmic actin filament structures and conducted further transcriptomics to identify significant inflammasomes. To our knowledge, this is the first study to compare blood-derived messenger RNA (mRNA) expression in patients with acute phase SSNS with heavy proteinuria and those in sustained remission without medication.

II. MATERIALS AND METHODS

1. Participants and their classification

This study commenced in 2016 and received approval from the Institutional Review Boards (IRB) at Yonsei University Severance Hospital for a 10-year duration involving human subjects (IRB approval number: 4-2016-0887). Following IRB approval, patient recruitment took place from 2016 to 2023.

Patients were recruited based on clinical criteria adapted in accordance with guidelines from the International Pediatric Nephrology Association (IPNA),[16] targeting those diagnosed with SSNS. The definition of INS required the presence of nephrotic-range proteinuria (urine protein to

creatinine ratio ≥ 200 mg/mmol [2 mg/mg] in a spot urine sample, or proteinuria ≥1,000 mg/m² per day in a 24-hour urine sample, corresponding to 3 + [300–1,000 mg/dL] or 4 + [≥1,000 mg/dL] by urine dipstick), hypoalbuminemia (serum albumin < 30 g/L), and edema.[16]

Over 85% of children older than one year presenting with the classic symptoms of INS exhibit SSNS, and if biopsy samples are taken, they frequently indicate minimal change disease (MCD).[1] Because of this, initial treatment is often administered instead of undergoing a kidney biopsy.[16] A diagnosis of SSNS is considered if the patient exhibits a positive response to corticosteroids at a standard dose within 4 weeks, leading to the disappearance of protein in the urine.[16] Patients who did not experience symptom improvement after 4 weeks of treatment were excluded from the study. Additional exclusion criteria included secondary causes of nephrotic syndrome, such as congenital nephrotic syndrome caused by genetic mutations, focal segmental glomerulosclerosis (FSGS), systemic lupus erythematosus, and glomerulonephritis.

Patients diagnosed with SSNS and participating in this study were classified as follows:

- Acute phase: The time of initial SSNS diagnosis or the time of proteinuria recurrence.
- Chronic remission phase: The point at which a patient with SSNS has maintained a state of normal urine without observable proteinuria and normal serum albumin, without drug therapy, for more than one year.

Based on this classification, patients underwent blood collection in either the acute or chronic remission phase. Blood collection was conducted a maximum of two times per patient: once during the acute phase and once during the chronic remission phase. If a patient did not meet the criteria for either the acute or chronic remission phase during the study period, blood collection was not performed.

Each blood collection session involved 7.5 cc of blood. The collected specimens were immediately dispatched to the laboratory. Of these, 5 cc was centrifuged to separate the serum and then stored in liquid nitrogen at -140 ℃. The remaining 2.5 cc of whole blood was placed in a PAXgene® blood RNA tube and stored in liquid nitrogen. The stored samples were anonymized and categorized into acute and chronic remission phase patients for preservation. The 5 cc of centrifuged serum was used to stimulate podocytes, while the 2.5 cc stored in the PAXgene® blood RNA tube was used for mRNA sequencing.

All patients recruited were pediatric and adolescent individuals aged 4 to under 18, considering the volume of blood collection. To ensure minimal bias from drug administration during the acute phase, blood samples were collected as soon after diagnosis as possible. Moreover, to mitigate patient-related bias in sampling, individuals on medication for conditions other than nephrotic syndrome or those exhibiting infection symptoms were excluded from the study.

For the chronic remission phase, patients who had undergone blood collection once during the acute phase and reached the chronic remission phase due to disease improvement within the study period were re-consented for an additional blood draw. Alternatively, for patients diagnosed with SSNS before the start of the study who maintained the chronic remission phase and visited the hospital for examination, blood collection was performed once during the chronic remission phase, without conducting acute phase blood collection. All participants, either directly or through their legal guardians, provided informed consent.

2. Whole blood experiments

2.1. Sample preparation and bulk-RNA sequencing

RNA sequencing was performed using whole blood samples from the two groups (acute phase and chronic remission phase), and a subsequent differentially expressed genes (DEGs) analysis was then conducted. Following treatment and incubation, patients' blood was suspended in RNAlater® (Invitrogen, Carlsbad, CA, USA) and stored at -20℃ to preserve RNA integrity. Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), with DNase digestion employed to prevent genomic DNA contamination. The quality and quantity of each RNA sample were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

mRNA isolated from total RNA using oligo-dT beads was used to construct a library using the NEXTFLEX® Rapid RNA-Seq Kit (PerkinElmer, Inc., Waltham, MA, USA). Additionally, small RNA-Seq libraries were prepared using the NEXTFLEX® Small RNA-Seq Kit (PerkinElmer Inc., Waltham, MA, USA) following the manufacturer's recommendations. Paired-end sequencing with a 100-bp read length was performed using the Illumina Nexseq550 (Illumina, San Diego, CA, USA) after quality control, and library quantity assessment using the Agilent 2100 Bioanalyzer.

2.2. Data analysis and statistical modeling

2.2.1. Principal component analysis (PCA) and the identification of DEGs

To analyze the complexity of high-dimensional datasets while preserving essential trends and patterns, we utilized PCA. This method was applied to the RNA sequencing expression profiles of individual samples to discern the general patterns among the genes.

Utilizing TopHat, htseq, and DESeq2 (version 1.10.0),[17] we conducted primary analysis of the high-throughput sequencing data. We aligned the raw FASTQ files to the UCSC hg19 reference genome using TopHat (version 1.4.0) and Bowtie (version 2.3.5).[18] After successfully aligning the sequence reads to the reference genome, we used HTSeq to count the reads mapped to each gene.

To mitigate potential biases stemming from library preparation protocols, sequencing platforms, and nucleotide compositions, a normalization process was subsequently implemented to address differences in read numbers across various sequencing runs. DEGs were identified by employing the DESeq2 package, which assumes a negative binomial distribution to estimate gene variance. We conducted pairwise comparisons between the two treated groups to determine statistically significant DEGs.

2.2.2. Potentially relevant gene selection

We performed a comparative analysis using patients' whole blood to explore differences in substances known to be associated with the inflammasome and related genes. Additionally, to identify genes implicated in the observed alterations, the DEGs were compared with data from previous studies and databases. PodNet, a database constructed from mouse podocyte data and enriched by expert curation and literature, is considered an instrumental resource for podocyte interactome analysis and the elucidation of podocyte expression data, despite being mouse-derived.[19]

2.2.3. Pathway enrichment analysis

We conducted an enrichment analysis of gene ontology (GO) terms for every DEG. The process involved hypergeometric testing to discern enriched GO terms by comparing the DEGs from each analysis to genes annotated within biological pathways in GO, applying a p-value threshold of < 0.05.

2.2.4. Protein-protein interaction (PPI) network analysis

The extensive STRING 9.1 network database (http://string91.embl.de/) serves as a comprehensive repository encompassing both direct (physical) and indirect (functional) PPI. It integrates data from diverse sources, including genomic context predictions, high-throughput experiments, co-expression analyses, and existing databases.[20] With information spanning more than 5 million proteins across 1,133 organisms, this database played a pivotal role in identifying PPIs linked to the genes, including DEGs from various comparisons.[20] In this study, we utilized the STRING database to explore the interactions between the proteins associated with the DEGs. To align with our gene sets represented by gene symbols, we converted the Ensembl protein identifiers in the original STRING database into gene symbols using mapping information in EntrezID. Finally, the PPI network was visualized and presented in graphical formats.

3. Stimulation experiments on podocytes using patient sera

3.1. Human podocyte cell culture

Conditionally immortalized human podocytes (AB8/23) were characterized and generously provided by Dr. Moin A. Saleem from the University of Bristol, Bristol, UK. The podocytes were maintained in RPMI 1640 medium (Gibco™) supplemented with 10% heat-inactivated fetal bovine serum (FBS), insulin-transferrin-selenium-pyruvate supplement (ITSP; Gibco™), and antibiotics. Media renewal occurred every 48 hours to maintain cellular freshness. To induce differentiation, podocytes were subjected to non-permissive conditions at 37℃ for a minimum of 2 weeks. Cell detachment from the culture dishes was facilitated using 0.05% trypsin.[21]

3.2. Evaluation of podocyte response after stimulation

Podocytes were allocated into four distinct groups for the experiment as follows:

- Group 1: Vehicle-treated podocytes (control)
- Group 2: Podocytes stimulated with acute phase sera
- Group 3: Podocytes stimulated with acute phase sera and treated with dexamethasone
- Group 4: Podocytes stimulated with chronic remission phase sera

Group 1 served as the control and received only the vehicle treatment. Group 2 was stimulated with acute phase serum, Group 3 was treated with a combination of acute phase serum and dexamethasone (DEX), and Group 4 was stimulated with chronic remission phase serum. The podocytes were acclimatized for a period of 4 hours before starting the main experiment. The entire process was replicated three times to ensure consistency.

The control group (Group 1) was incubated in 1% bovine serum albumin (BSA), a commonly used carrier protein, as the vehicle alone. Human podocytes in Groups 2 and 4 were exposed to patient serum in a serum-free RPMI 1640 medium at a temperature of 37℃. Since there was no existing literature to reference for this specific matter, the exposure duration to patient serum was determined through preliminary experiments. These preliminary experiments varied the exposure time of podocytes to serum and revealed that too short an exposure did not induce significant changes in the podocytes, whereas too lengthy an exposure reduced the survival rate of the podocytes, leading to errors in comparisons. Consequently, the appropriate exposure duration was ultimately established at 18 hours for this experiment.

For Group 3, podocytes were treated identically to Groups 2 and 4, with the addition of DEX sourced from Sigma-Aldrich (Saint Louis, MO, USA). DEX was dissolved in distilled water following the manufacturer's protocols. Previous publications have conducted detailed quantitative analyses with varying DEX concentrations, determining that a range from 10^{-7} to 10^{-5} M reflects the therapeutic levels found *in vivo*.[22] However, treating podocytes exposed to patient serum with DEX is a novel aspect of this study. It was determined that using a concentration of 1 mM, higher than the concentration of DEX previously employed, would yield a more definitive therapeutic effect. Therefore, 1 mM was selected for the treatment in this study.

3.3. Visualization of intracytoplasmic actin filaments

To determine whether patient serum influences the intracytoplasmic actin filaments of podocytes, a key feature of SSNS development, these structures were stained and examined under a fluorescence microscope. Additionally, a comparative experiment was conducted to investigate the impact of DEX treatment to determine if there is an improvement. After completing the procedures for Groups 1 to 4, as described in section 3.2 above, podocytes were assessed.

Cell fixation was accomplished using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at ambient temperature. For permeabilization, 0.3% Triton X-100 in PBS was utilized for 10 minutes at the same temperature. After rinsing with PBS, the staining procedure was performed using rhodamine-phalloidin (Invitrogen) at a dilution of 1:500 for 30 minutes at room temperature. Post-staining washes were done with PBS, followed by 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) staining for the nuclei at a dilution of 1:5,000. The samples were then mounted and observed for actin filaments and nuclei using fluorescence microscopy.

To correct for biases such as differences in the background during microscopic imaging and to enhance accuracy for statistical analysis, experiments were performed in triplicate. The acquired images were analyzed using Image J software (version 1.50i; National Institutes of Health, Bethesda, MD, USA), counting the actin filaments in each cell using the point-counting technique.

3.4. Transcriptomic analysis of podocytes

Utilizing the stimulated podocytes, the same methodology for RNA sequencing, data analysis, and statistical modeling was applied. The procedures mirrored those outlined in sections 2.1 and 2.2 above.

III. RESULTS

1. Experiments with patient whole blood samples

1.1. PCA

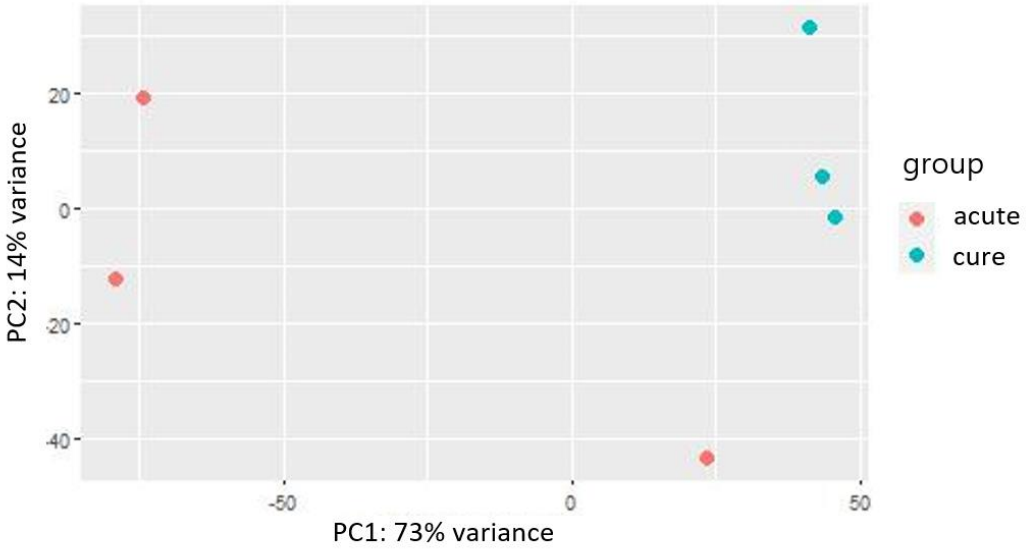
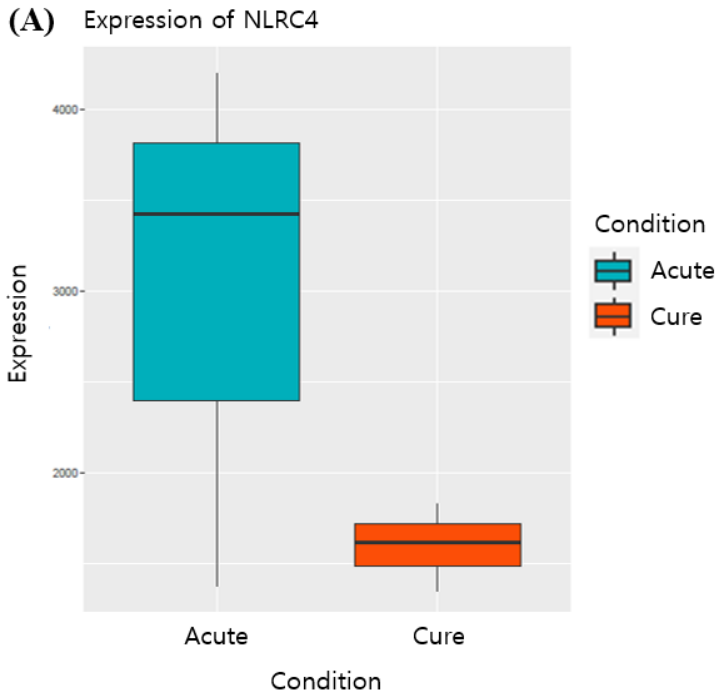


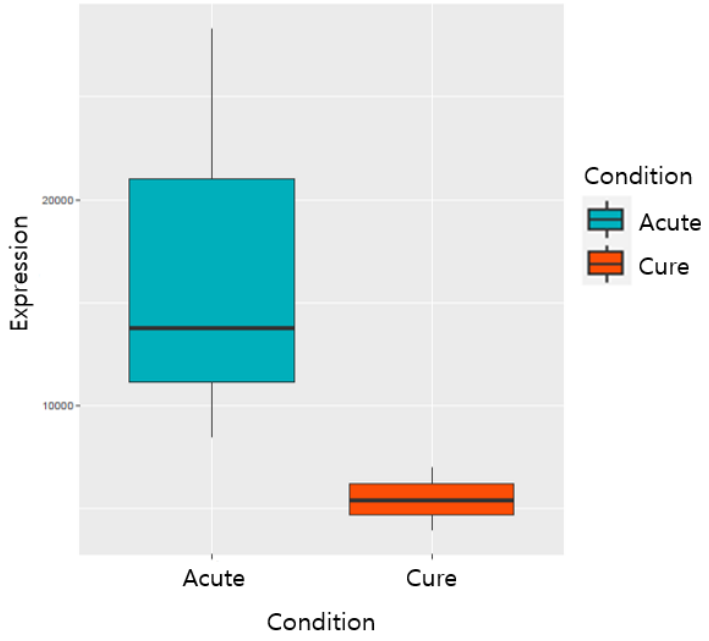
Figure 1. Whole-transcriptome principal component analysis (PCA) using patients' whole blood. The acute phase (red) and chronic remission (green) groups are represented in different regions. Two major principal components (PC) account for 87% of the variance, with PC1 explaining 73% and PC2 explaining 14%. Abbreviations: PC, principal components; acute, acute phase patient group; cure, chronic remission phase patient group.

PCA was initially conducted using whole blood from patients in the acute and chronic remission phases. The PCA results revealed a distinct separation between the two groups, with over 87% of their variance explained by principal components 1 (PC1) and PC2 (Figure 1). A total of 3,554 DEGs were identified between the two groups (adjusted p-value < 0.02). The clear separation in the PCA indicates significant differences in gene expression patterns between the acute and chronic remission phases.

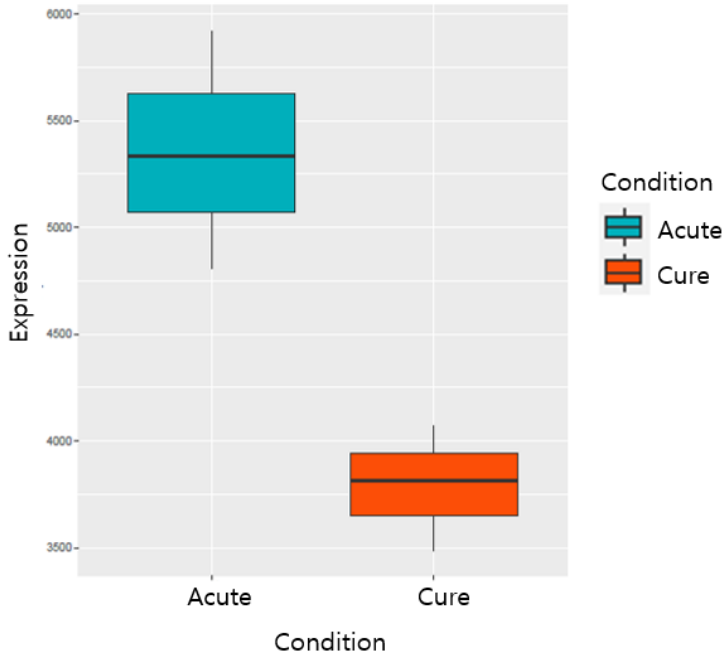
1.2. Potentially relevant gene selection



(B) Expression of NAIP



(C) Expression of NFKB1



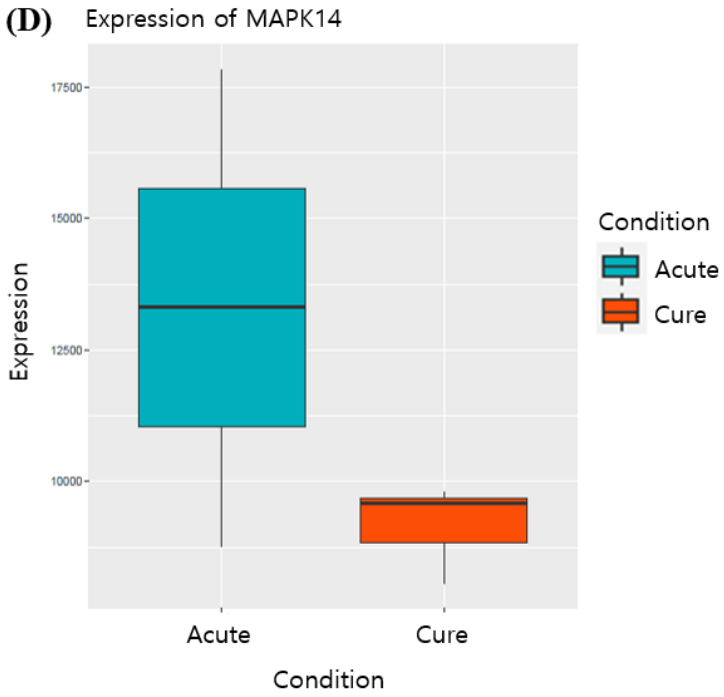
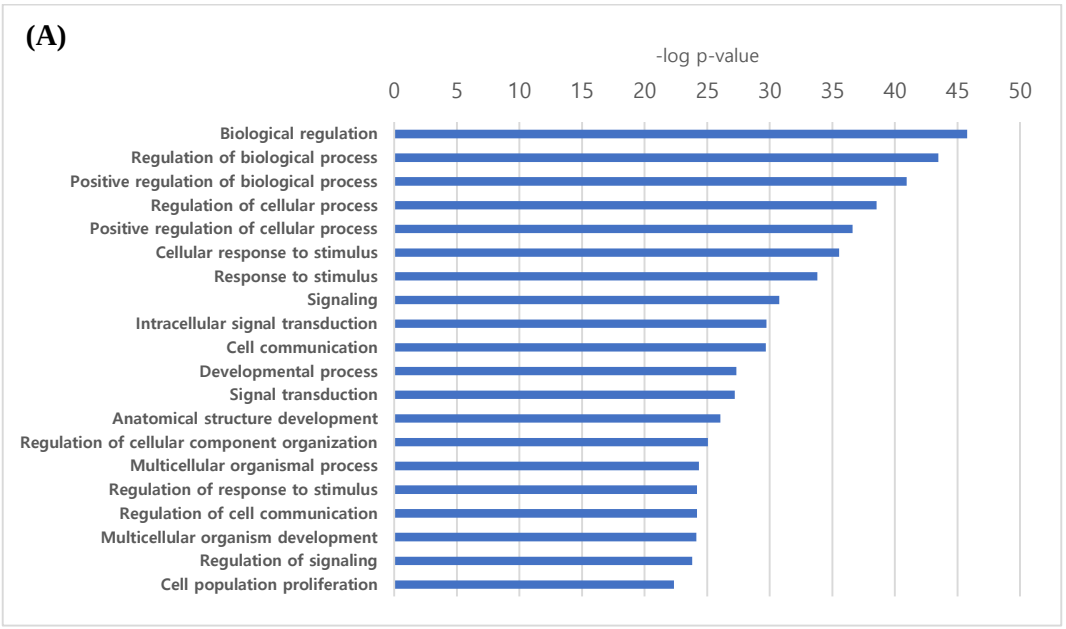


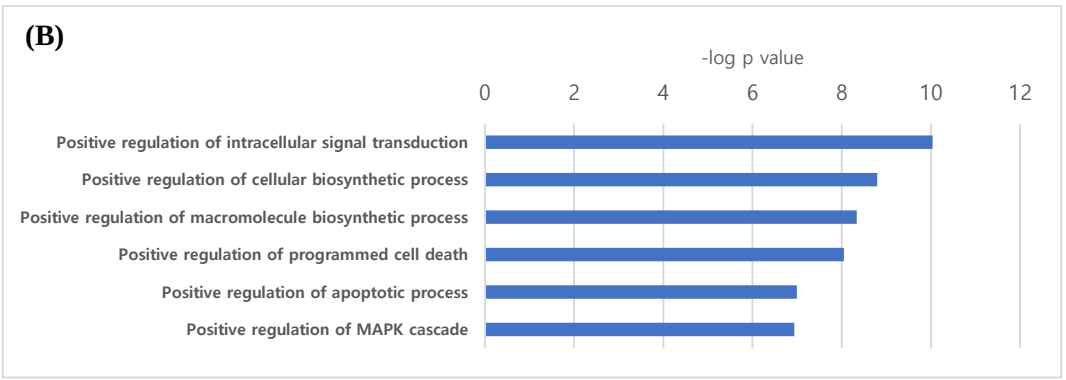
Figure 2. Expression analysis of four genes known to be associated with the inflammasome in the two patient groups: (A) *NLRC4*, **(B)** *NAIP*, **(C)** *NFKB1*, and **(D)** *MAPK14*. Abbreviations: acute, acute phase patient group; cure, chronic remission phase patient group; *NLRC4*, Nod-like receptor family caspase activation and recruitment domain (CARD) containing 4; *NAIP*, nucleotide-binding domain and leucine-rich repeat containing family apoptosis inhibitory protein; *NFKB1*, human nuclear factor-kappa B1; *MAPK14*, mitogen-activated protein kinase 14.

We used whole blood from patients in the acute and chronic remission phases to investigate the expression differences of four genes associated with the inflammasome: *NLRC4*, nucleotide-binding domain and leucine-rich repeat containing family apoptosis inhibitory protein (*NAIP*), nuclear factor-kappa B1 (*NFKB1*), and mitogen-activated protein kinase 14 (*MAPK14*) (Figure 2A-D). These genes were relatively overexpressed during the acute phase, with reduced expression as the condition returned to a chronic remission phase, with these differences being statistically significant. Concurrent analysis of *NLRP3* revealed no statistically significant differences between

the two groups.

1.3. Pathway enrichment analysis





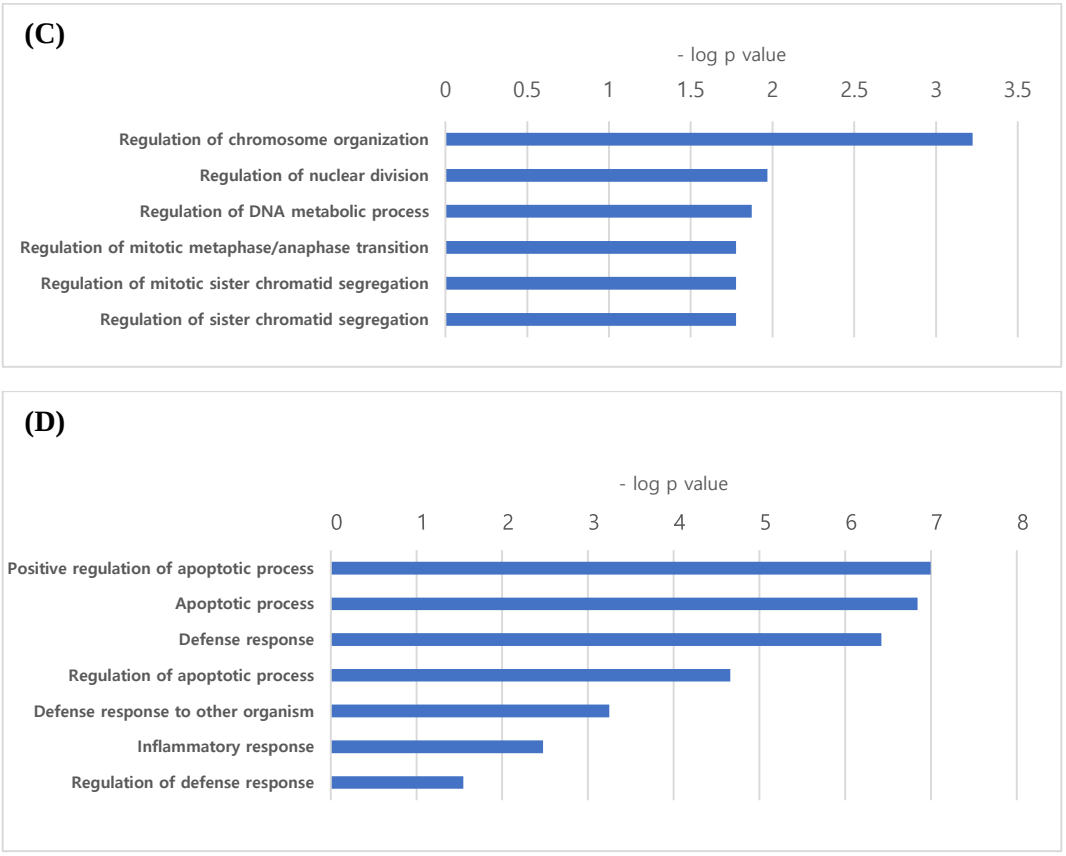


Figure 3. Gene ontology (GO) enriched pathway analyses. (**A**) Enriched pathways among the most distinct DEGs between acute and chronic remission phase groups. (**B**) Enriched pathways among up-regulated DEGs in the acute phase group. **(C)** Enriched pathways among down-regulated DEGs in the acute phase group. (**D**) Enriched pathways related to "inflammasome" or "podocyte cytoskeleton" between acute and chronic remission phase groups. Abbreviations: DEGs, differentially expressed genes.

To delineate the clinical relevance of the DEGs identified between patients in the acute phase and those in the chronic remission phase, we performed GO enrichment analyses (Figure 3). Among the 3,554 DEGs identified, the most significantly affected pathways included biological regulation, cellular process regulation, cellular response to stimulus, signaling, cell communication, and

developmental processes (Figure 3A). The analyses revealed that DEGs with increased activity were associated with the regulation of intracellular signal transduction, cellular biosynthetic processes, macromolecule biosynthetic processes, programmed cell death (PCD), apoptotic processes, and the MAPK cascade (Figure 3B). Conversely, down-regulated pathways were linked to chromosome organization, nuclear division, DNA metabolism, transition from mitotic metaphase to anaphase, and segregation of mitotic sister chromatids (Figure 3C). Additionally, when exploring pathways related to inflammasomes and the podocyte cytoskeleton, we pinpointed three key pathways: apoptosis, defense response, and inflammatory response (Figure 3D).

1.4. PPI network

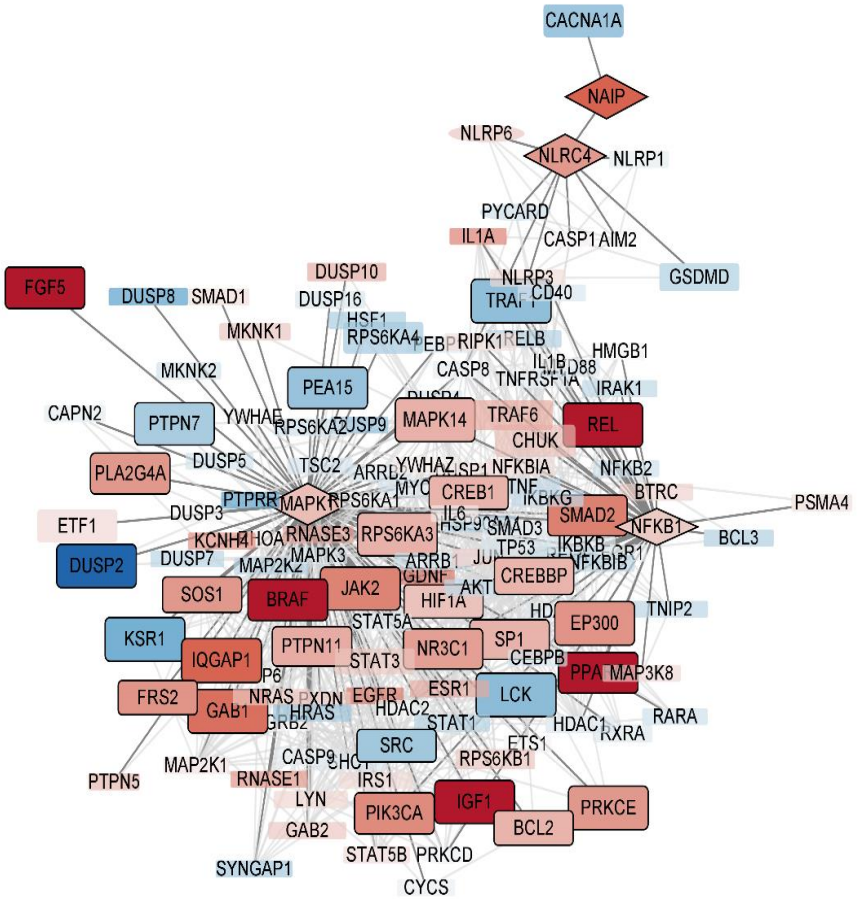


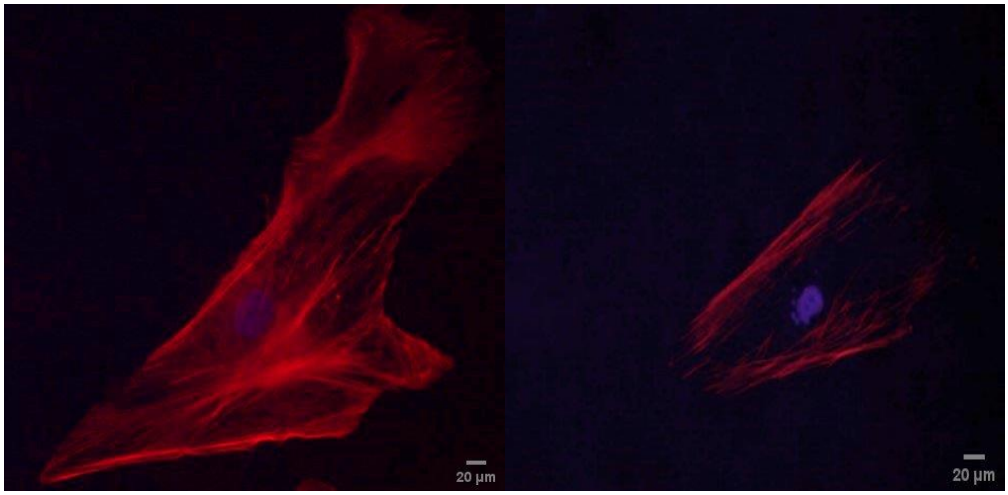
Figure 4. Protein-protein interaction (PPI) network of the 3,554 differentially expressed genes (DEGs) identified between the acute phase and chronic remission phase groups. Red and blue nodes represent genes that are up-regulated and down-regulated during the acute phase, respectively. The intensity of the color corresponds to fold change values; that is, the greater the difference, the more vivid the color. The size of each node is proportional to the -log p-value, indicating the statistical significance of the genes.

In the PPI network analyses, we explored the intricate dynamics among the 3,554 DEGs (Figure 4), revealing a complex network of interactions among these genes, with a pronounced hub of connectivity surrounding the *NFKB1*, *MAPK14*, and *NLRC4* genes. Genes implicated in inflammasome activity are indicated with circles, while key genes such as *NFKB1*, *MAPK14* and *NLRC4* are marked with diamonds. These pivotal pathways are represented as being directly interacted with by genes connected by black lines, and PPIs without diamond-shaped genes are denoted with gray lines. Notably, *NLRC4* and *NAIP* are closely interconnected, and despite their more subdued links to other genes, their involvement in the network signifies pertinent interactions.

2. Stimulation experiments on podocytes using patient sera

2.1. Visualization of intracytoplasmic actin filaments

(A) **(B)**



(C) **(D)**

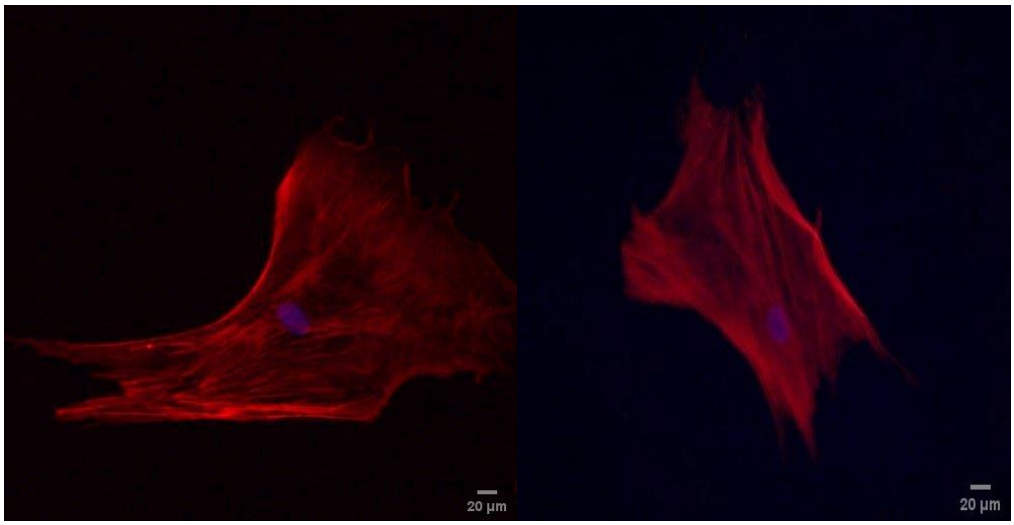
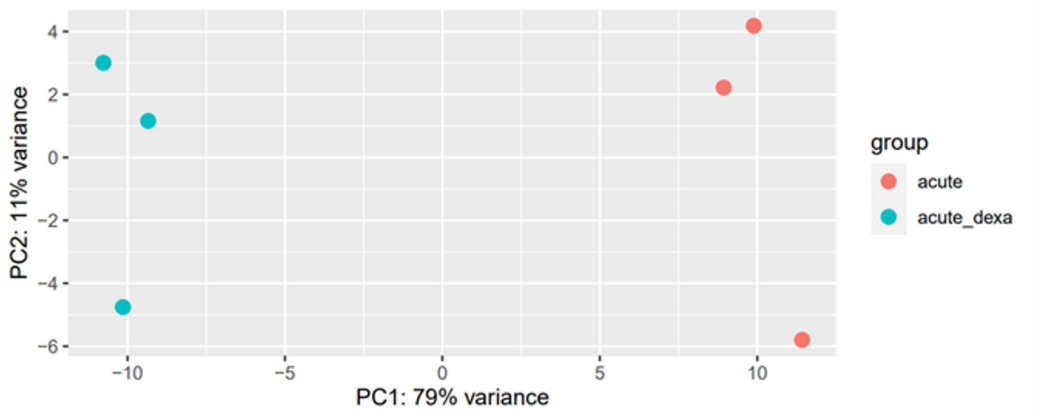


Figure 5. Intracytoplasmic actin filaments (red) revealed by rhodamine-phalloidin staining. (**A**) Vehicle-treated control podocytes, (**B**) podocytes stimulated with acute phase serum, (**C**) podocytes exposed to acute phase serum and treated with DEX, and (**D**) podocytes stimulated with chronic remission phase serum. Nuclei are stained blue by DAPI. Abbreviations: DEX, dexamethasone; DAPI, 4',6-diamidino-2-phenylindole.

Subsequent analysis was performed using immunofluorescence microscopy to examine the effects on intracytoplasmic actin filaments. Cells stimulated with acute phase serum displayed a reduction in intracytoplasmic actin filaments compared to the vehicle-treated control group and those stimulated with chronic remission phase serum. Furthermore, the addition of DEX to the acute phase serum-stimulated cells resulted in a noticeable restoration of actin staining clarity, indicating a mitigating effect of DEX on cytoskeletal alterations induced by the acute phase serum (Figure 5A-D).

2.2. PCA

(A)



(B)

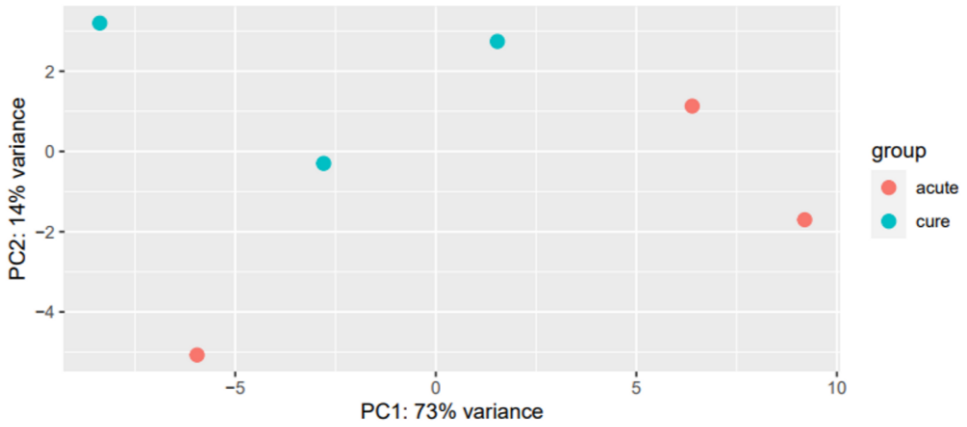
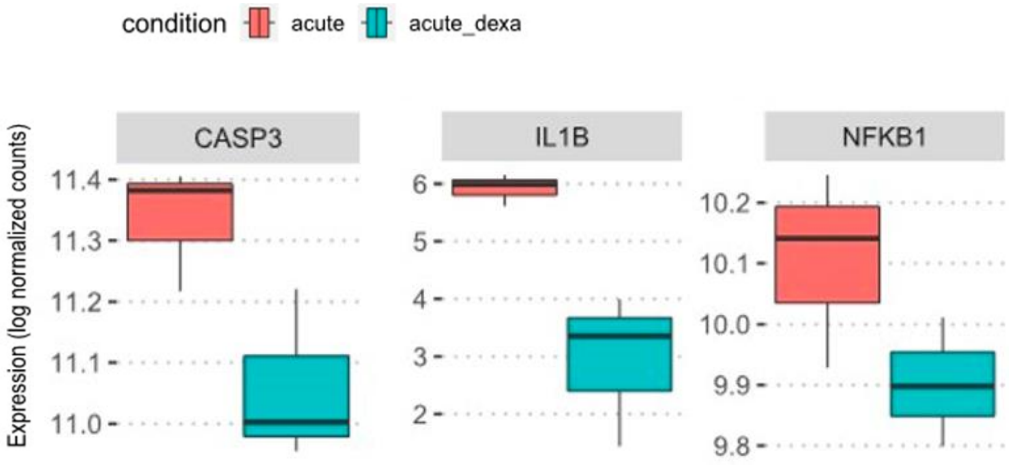


Figure 6. Principal component analysis (PCA) between the podocyte groups. Podocytes stimulated with acute phase serum (red) and (**A**) acute phase serum plus DEX (green) or (**B**) chronic remission phase serum (green). Two major PCs account for 90% and 87% of the variance, respectively, with (**A**) PC1 explaining 79% and PC2 explaining 11%, and (**B**) PC1 explaining 73% and PC2 explaining 14%. Abbreviations: PC, principal components; DEX, dexamethasone; acute, acute phase patient serum group; cure, chronic remission phase patient serum group.

PCA was utilized to differentiate between the various podocyte conditions. In the PCA plot (Figure 6A), a clear distinction is observed when contrasting podocytes subjected to acute phase serum against those treated with acute phase sera and DXA. Combined, PC1 and PC2 account for 90% of the variance. This comparison also revealed a total of 2,153 DEGs with an adjusted p-value threshold < 0.05. Conversely, in Figure 6B, podocytes treated with acute phase serum were compared to those treated with serum from the chronic remission phase. The PCA plot in this scenario did not exhibit a pronounced separation as seen in the previous comparison. Correspondingly, the number of DEGs detected was considerably lower, with only 574 genes meeting the selection criteria.

2.3. Potentially relevant gene selection

(A)



(B)

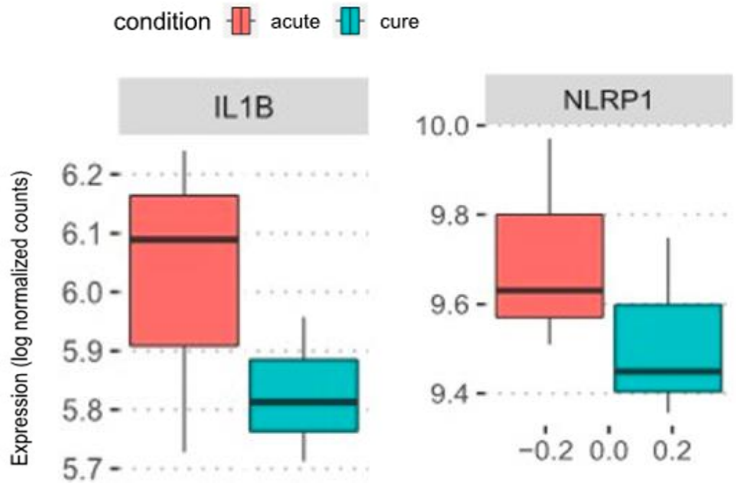


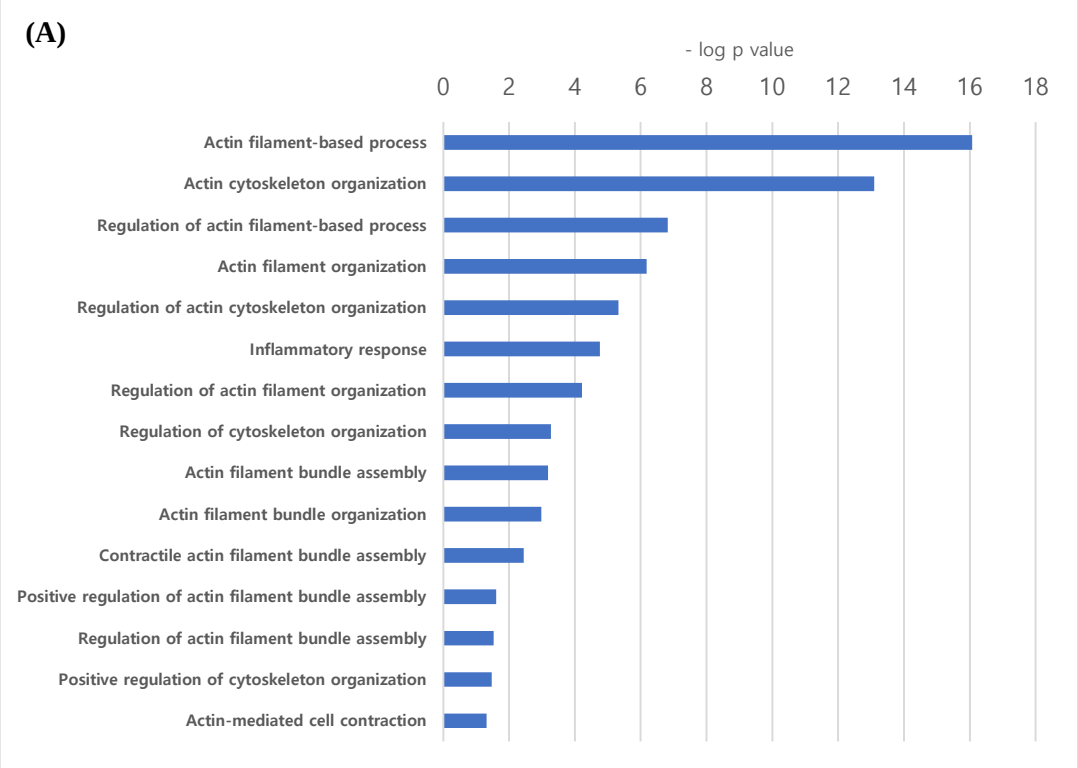
Figure 7. Expression analysis of genes associated with the inflammasome. (**A**) This analysis contrasts the gene expression profiles in podocytes after acute phase serum stimulation with those in podocytes exposed to acute phase serum and DEX intervention. (**B**) A comparison of gene expression in podocytes exposed to acute phase serum versus podocytes treated during the chronic remission phase. Abbreviations: DEX, dexamethasone; *CASP3*, caspase-3; *IL1B*, interleukin-1β; *NFKB1*, human nuclear factor-kappa B1; *NLRP1*, Nod-like receptor family pyrin domain containing 1.

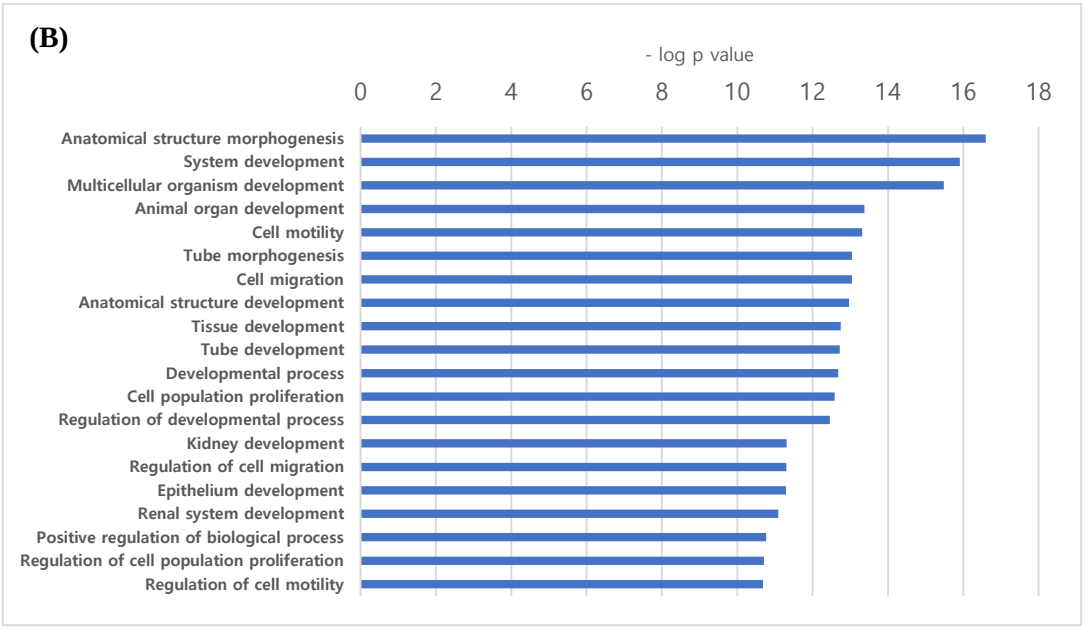
In this study, we assessed the DEGs associated with the inflammasome in podocyte groups. Initially, the gene expression profiles were compared between a group of podocytes stimulated with acute phase serum and a group that received acute phase serum and treatment with DEX. Through this comparison, we obtained data on three genes: caspase 3 (*CASP3*), IL-1β (*IL1B*), and *NFKB1* (Figure 7A). These genes exhibited up-regulated expression when stimulated with acute phase serum and showed down-regulation following DEX treatment. The observed differences were statistically significant.

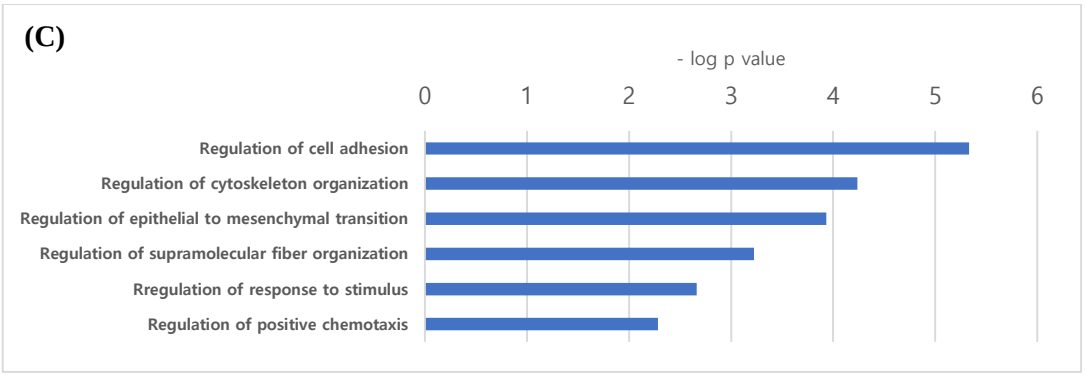
Comparing the gene expression between podocytes stimulated with acute phase serum and those stimulated with chronic remission phase serum revealed a statistically significant variation in *IL1B* levels. Although *NLRP1* did not exhibit statistically significant differences, it showed a trend

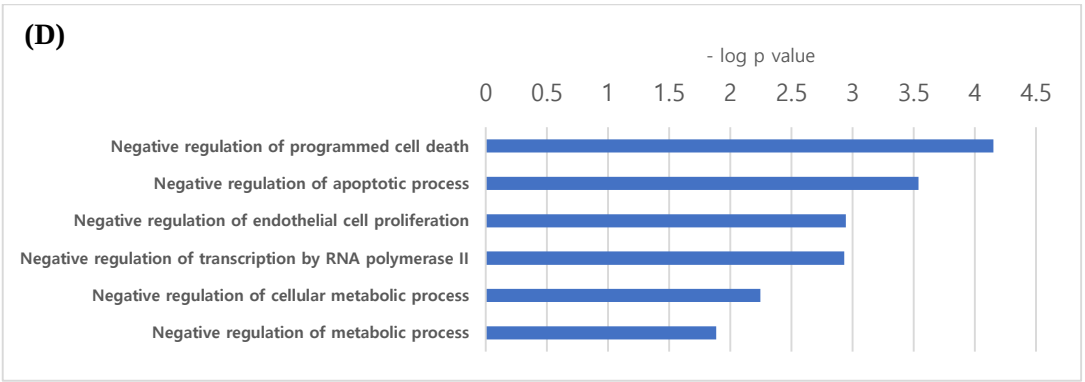
of higher activity in podocytes stimulated with acute phase serum, similar to *IL1B* (Figure 7B). In these podocyte experiments, *NLRC4* and *NLRP3* did not show significant differences.

2.4. Pathway enrichment analysis









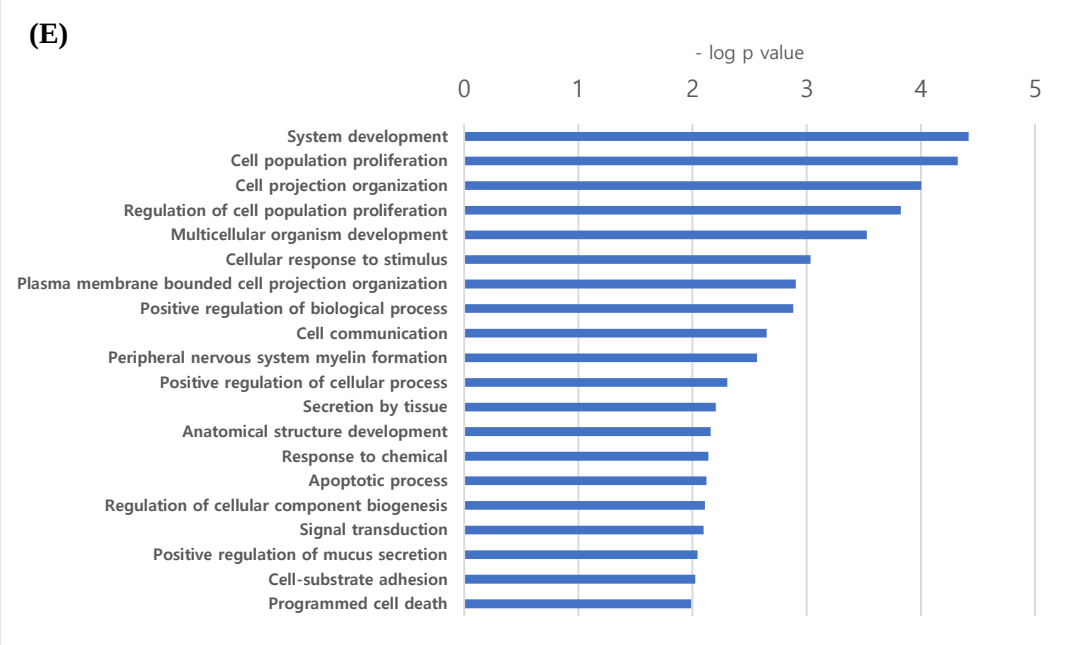


Figure 8. Enriched pathway analyses based on gene ontology (GO). (A) Enriched pathways related to "inflammasome" or "actin" between podocytes stimulated with acute phase serum and those exposed to acute phase serum plus DEX treatment. DEGs overlapping with the PodNet were used for following GO analyses: enriched pathways among (**B**) the most distinct DEGs in podocytes stimulated with acute phase serum, (**C**) up-regulated DEGs in podocytes stimulated with acute phase serum, (**D**) down-regulated DEGs in podocytes stimulated with acute phase serum compared to the group treated with DEX. (**E**) Enriched pathways among DEGs that showed the most significant differences in podocytes stimulated with acute phase serum compared to those stimulated with chronic remission phase serum. Abbreviations: DEX, dexamethasone; DEGs, differentially expressed genes.

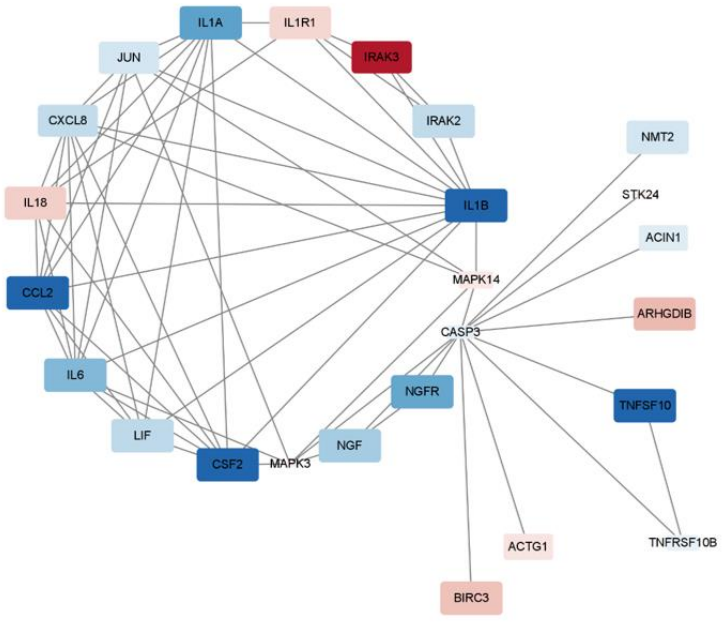
We conducted GO enrichment analyses to elucidate the clinical significance of the DEGs in podocytes stimulated with patient serum (Figure 8). Among the 2,153 DEGs identified between groups treated with acute phase serum and those additionally treated with DEX, the most significantly impacted pathways were primarily associated with actin filament and cytoskeleton-related processes and organization, as well as inflammatory responses (Figure 8A).

Out of the 2,153 DEGs, 79 were found to overlap with the PodNet database, and these genes were subsequently utilized for further analyses. The pathways most significantly affected by the addition of DEX included anatomical structure morphogenesis, system and organ development, and cell motility and migration, which also encompassed kidney and renal system development (Figure 8B). Genes up-regulated in podocytes upon stimulation with acute phase serum, without DEX treatment, were involved in processes regulating cell adhesion, cytoskeleton organization, epithelial to mesenchymal transition, supramolecular fiber organization, response to stimuli, and positive chemotaxis (Figure 8C). Conversely, pathways showing a decrease in activity when treated concurrently with DEX were related to PCD, the apoptotic process, endothelial cell proliferation, transcription by RNA polymerase II, and metabolic processes (Figure 8D).

Among the 574 DEGs identified between groups treated with acute phase serum and those treated with chronic remission phase serum, 17 were found to overlap with the PodNet. GO analysis of these two groups revealed involvement in system development, cell population proliferation, projection organization and communication, cellular response to stimulus, and apoptotic processes (Figure 8E).

2.5. PPI network

(A)



(B)

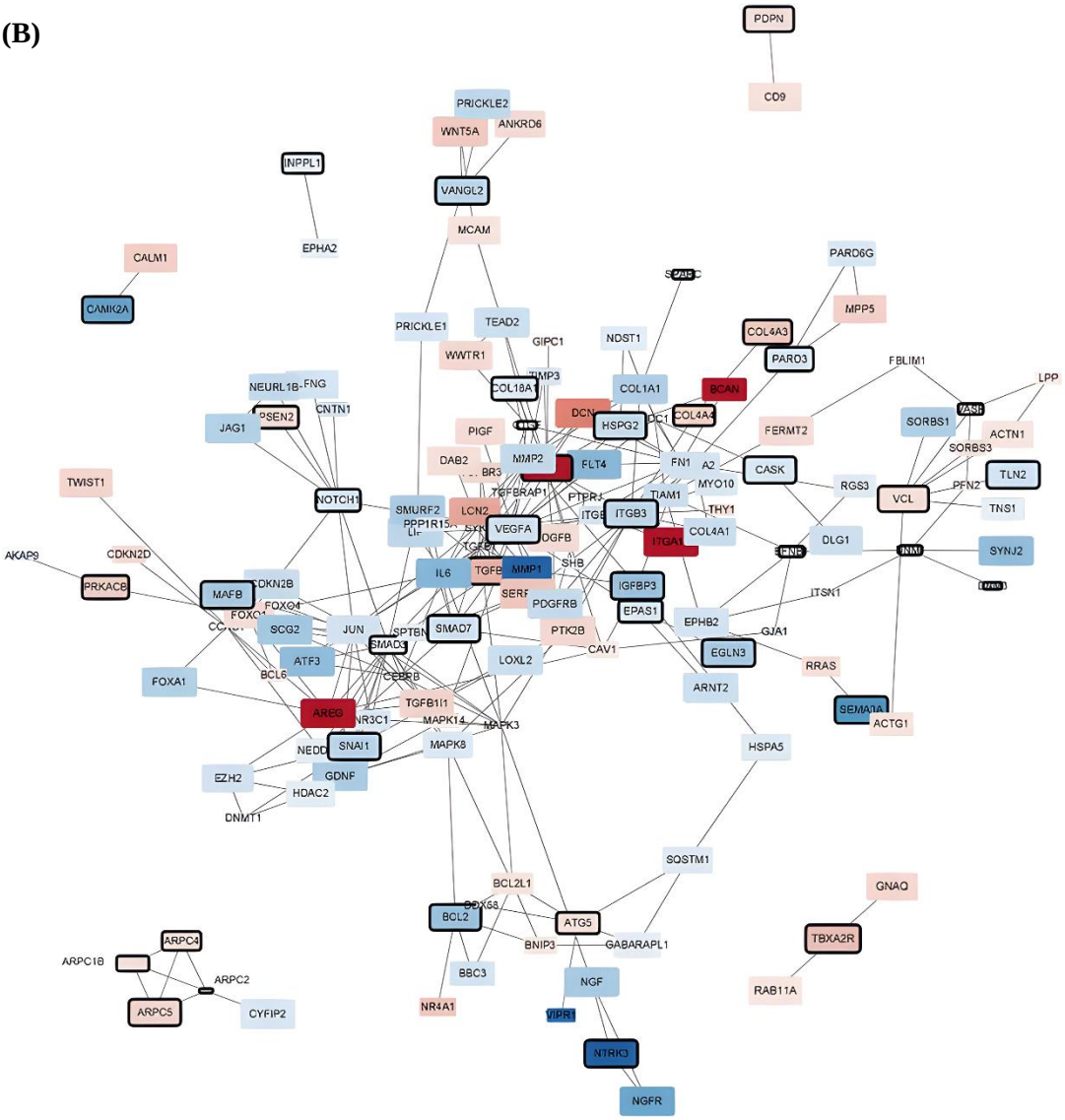


Figure 9. Protein-protein interaction (PPI) network of the differentially expressed genes (DEGs) of the podocyte group stimulated with acute phase sera and the group treated with acute phase sera plus dexamethasone. (A) A PPI diagram delineating the interactions among genes known to be associated with the inflammasome within the DEGs. **(B)** A PPI diagram illustrating interactions among 79 genes overlapping with PodNet within a subset of the DEGs network.

We established the PPI networks for DEGs elicited from cells stimulated with acute phase serum, as well as for cells concomitantly treated with DEX. Figure 9A presents a PPI network highlighting the relationships among DEGs implicated in inflammasome activity. The results of the PPI diagram revealed that *CASP3* and *IL1B*, known to be associated with the inflammasome, play crucial roles in interactions with other genes. Figure 9B details a PPI network focusing on a specific subset of 79 DEGs that intersect with the PodNet database, illuminating their potential collaborative roles. In this figure, the colors represent whether podocytes stimulated with acute phase serum showed up-regulated (red) or down-regulated (blue) gene expression compared to those exposed to acute phase serum and treated with DEX, with bold borders highlighting podocyte-related genes.

IV. DISCUSSION

In the burgeoning field of nephrology, inflammasomes have become a point of interest due to their potential role in the pathogenesis of various renal pathologies. Since the initial discovery of inflammasomes, [6] research has intensified to elucidate their connection with kidney diseases. Many renal disorders are now believed to be directly immunologically mediated by or originate from immune system modifications secondary to structural kidney damage, such as that observed in diabetic nephropathy. [23-25]

Research efforts have been primarily directed towards adult kidney diseases, which represent a significant burden due to their prevalence. Among the various inflammasomes, NLRP3 has emerged as the most extensively studied over the past decades, [26] due to the compelling evidence associating it with a wide range of pathological conditions. This has subsequently influenced the direction of research, with a focus on NLRP3 across a range of diseases affecting the kidneys and other organs.

Regarding research on INS and inflammasomes published to date, studies such as Lucafò et al. [27] have found that hypomethylation of the NLRP3 promoter could be used to distinguish between INS patients who are resistant to corticosteroids and those who are sensitive. Wang et al. [28] observed elevated expression of the inflammasome product IL-1β in renal biopsy tissues from groups with MCD, mesangial proliferative glomerulonephritis, and FSGS, compared to a control group.

Additionally, in a mouse model of nephrotic syndrome, moderate tubulointerstitial nephritis and fibrosis were observed, along with increased mRNA and protein levels of NLRP3, apoptosis-related speck-like protein (ASC), and pro-caspase-1/caspase-1.[29] Another study using mouse podocytes found that cluster of differentiation 36 (CD36) plays a crucial role in promoting podocyte apoptosis by activating the NLRP3 inflammasome in nephrotic syndrome.[15] However, compared to other diseases, the research conducted on this topic is still relatively scarce.

In the context of INS, specifically SSNS, which predominantly impacts children but also occurs in adults, it was essential to evaluate the involvement of inflammasomes in its pathogenesis. By leveraging insights from adult renal disease and broader disease-related studies, the hypothesis was that NLRP3 is a key etiological component. Contrary to this hypothesis, as will be discussed later, the findings from the present investigation indicate that NLRP3 may not play the central role initially presumed.

This study employs several innovative methods. First, it compares the acute phase blood from patients with SSNS exhibiting significant proteinuria to blood from patients in a chronic remission phase. These chronic remission phase patients have previously been diagnosed with SSNS but have been asymptomatic without any recurrence of proteinuria or the need for medication for over a year, making their condition comparable to that of healthy individuals. This type of comparative analysis is unprecedented in existing research. Additionally, the study tackles the challenging task of culturing human podocytes, a process that is more complex than culturing other non-podocyte cells or podocytes derived from non-human species such as mice. It breaks new ground by stimulating these cultured human podocytes with serum from patients suffering from SSNS.

In our comparative analysis of the acute and chronic remission phases in patients with SSNS, we identified significant differential expression in 3,554 DEGs. Among these, a novel expression pattern of genes associated with the NAIP/NLRC4 inflammasome and related pro-inflammatory cytokines was discovered. Contrary to our initial hypothesis, the expression levels of genes associated with the NAIP/NLRC4 inflammasome were significantly elevated during the acute phase and decreased during the chronic remission phase. Interestingly, no significant differential expression was noted in the *NLRP3* gene. This suggests a distinct regulatory mechanism of the NAIP/NLRC4 inflammasome in the pathophysiology of SSNS, which may have implications for therapeutic strategies (Figure 2).

Existing literature establishes that while both NAIP/NLRC4 and NLRP3 inflammasomes are integral components of the innate immune system, the assembly of NAIP/NLRC4 inflammasomes requires the presence of NAIP proteins.[30] These proteins serve as receptors to bacterial ligands, recruiting NLRC4 and forming the inflammasome complex.[30] In contrast, the activation of NLRP3 can be induced by various mechanisms that converge on mitochondrial dysfunction and the production of ROS, rather than the direct ligand-receptor interactions observed in NAIP/NLRC4.[31] Functionally, the NAIP/NLRC4 inflammasome primarily responds to bacterial proteins such as flagellin and components of the type III secretion system, showing a robust response to specific bacterial infections.[32-36] On the other hand, the NLRP3 inflammasome responds to a broader array of stimuli, including microbial components, environmental irritants, and endogenous danger signals, acting as a more general sensor in the immune response.[37] It has been reported that the NAIP/NLRC4 inflammasome is involved in detecting bacterial components, leading to activation that can result in the release of pro-inflammatory cytokines and cell death.[38,39]

Our GO pathway enrichment analysis revealed that during the acute phase, there is a significant up-regulation of pathways related to PCD and apoptotic processes, as well as cellular biosynthetic processes and cell proliferation/division/differentiation regulation through the MAPK signaling pathway (Figure 3B). The gene *NFKB1*, which we found to be in the same context as NLRC4/NAIP, is known to promote apoptosis.[40-43] While the exact functions of the concurrently discovered *MAPK14* gene remain largely unexplored, its identified involvement in the MAPK pathway suggests a connection to the regulation of cell apoptosis.[44]

Our podocyte experiment results align with these observations, demonstrating a marked reduction in gene expression levels of *CASP3*, *IL1B*, and *NFKB1* in podocytes stimulated with acute phase serum and DEX compared to those exposed to acute phase serum only (Figure 7A). Building on existing research, the NAIP/NLRC4 inflammasome serves as a key component of the immune response by mediating pro-inflammatory cytokine processing such as IL-1β.[6,45] Its activation can enhance the production of pro-inflammatory cytokines, triggering inflammatory responses and cell death.[6,45,46] Caspase-3 is a pivotal protease in the process of apoptosis, playing a significant role in the orchestrated dismantling of cells during PCD.[47] Beyond its traditional role, caspase-3 is also intertwined with the inflammasome, influencing both the generation of cell cytokines and cell death pathways.[48]

Additionally, our experiments revealed that intracytoplasmic actin filaments were damaged

upon stimulation with serum from the acute phase, which showed signs of recovery with DEX treatment (Figure 5). This suggests that the damage mechanism affects the structure of podocytes, particularly the actin cytoskeleton and that DEX is capable of partially reversing this damage. In acute phase serum stimulation, the up-regulated DEGs that overlapped with the PodNet were highly enriched for cytoskeleton organization and supramolecular fiber organization-related pathways, while down-regulated DEGs were associated with processes such as PCD, apoptotic processes, and metabolic processes (Figure 8C, 8D).

In this study, significant activation of the *NFKB1* gene, associated with the NF-κB protein, was observed during the acute phase in both *in vivo* studies of patients with SSNS and *in vitro* podocyte stimulation experiments. NF-κB is a key transcription factor that regulates various biological processes such as immune responses, inflammation, cell growth, and apoptosis.[49] In response to inflammatory signals, NF-κB is released from IκB proteins (inhibitors of NF-κB) in the cytoplasm, enabling its translocation into the nucleus to modulate gene expression.[50] Within the recognized framework of the NAIP/NLRC4 inflammasome, NF-κB activation is mediated by established components such as ASC proteins.[51] This activation cascade subsequently modulates the expression of pro-caspase-1, an essential factor for the functional engagement of the NAIP/NLRC4 inflammasome.[52] Activation of the NAIP/NLRC4 inflammasome leads to the secretion of proinflammatory cytokines such as IL-1β and IL-18, which in turn induce NF-κB activation, creating a positive feedback loop that enhances NAIP/NLRC4 inflammasome activation.[52] Thus, the interaction between NF-κB and the NAIP/NLRC4 inflammasome modulates various biological processes, including inflammation, cell death, and immune responses. Consequently, the results of this study suggest that the interplay between NF-κB and the NAIP/NLRC4 inflammasome may play a role in the pathogenesis of SSNS.

In the context of nephrotic syndrome, the gene expressions illustrated in the PPI network (Figure 4) are interconnected and associated with the NAIP/NLRC4 inflammasome, offering a specific aspect distinct from NLRP3 in the mechanistic interpretation of INS. Additionally, the increased expression of NAIP/NLRC4 inflammasomes, NF-κB, the MAPK14 pathway, caspase-3, and their intricate interrelations may imply the important role of maintaining a balance between pro-inflammatory and anti-inflammatory signaling for podocyte homeostasis. Disruptions in this balance may lead to pathological changes in podocytes, potentially explaining the higher incidence of SSNS observed in pediatric patients who are more susceptible to infections.[1,53] As previously mentioned,

although the NAIP/NLRC4 inflammasome was originally identified as being activated by bacterial infection,[32-36] other factors have also been studied.

Canna SW et al. reported a patient with macrophage activation syndrome due to a novel *de novo* mutation in *NLRC4*.[54] This mutation led to a gain of function in the NLRC4 protein, resulting in increased production of IL-1β and IL-18 and enhanced pyroptosis in macrophages.[54] Their study suggested a possible association between a unique inflammasome defect and autoinflammatory disease in the patient.[54] Although our nephrotic syndrome patients are not directly related to such autoinflammatory diseases, elucidating the role of these endogenous factors remains crucial to understanding the observed individual variability in inflammasome responses and their subsequent contribution to inflammasome-mediated podocyte injury pathways. Therefore, further follow-up studies related to this matter are warranted.

The limitations of this study can be attributed to several factors. Firstly, a small number of patients participated in the study, and the quantity of patient blood available for experimentation was quite limited. INS is not a common disease, which results in a lower number of diagnosed patients. Even after diagnosis, it took several months to confirm patients who met the criteria for SSNS, and it was challenging to time the collection of serum samples during the acute phase when patients experienced a relapse of proteinuria. Furthermore, not all patients and guardians consented to participate in this study, and even when consent was obtained, it was impractical to expect patients to visit the hospital daily. Thus, blood samples were usually taken during routine visits, which were infrequent. This was especially true for patients in the chronic remission phase, who visited the hospital less often than those in the acute phase. Consequently, obtaining consent and scheduling the next visit significantly delayed the process, limiting the number of samples collected within the study period. Due to the limited quantity of blood, it was challenging to conduct experiments on a large scale multiple times.

Once the patient's blood was collected, great care was taken to immediately centrifuge and store it at -140℃ to prevent hemolysis or damage. However, despite these precautions, there were instances where blood samples were deemed unusable for experiments due to inherent issues with the blood itself. The variability in the timing of blood collection could also contribute to differences between recently collected samples and those obtained earlier. The scarcity of existing research related to inflammasomes in SSNS further complicated the process of setting up podocyte stimulation experiments.

Since the majority of pediatric INS patients are steroid-sensitive and most of them are diagnosed with MCD, the response to steroid therapy is considered more indicative of long-term prognosis than conducting a renal biopsy.[55,56] Accordingly, in this study, renal biopsies were not initially performed in pediatric patients with nephrotic syndrome, following the definition set by the IPNA guidelines[16] adopted for this study. However, for this reason, it cannot be ruled out that a very small number of the SSNS patients included may have been those with FSGS.

To minimize bias during the acute phase, blood sampling was primarily carried out on patients who were newly diagnosed with INS. In other cases, efforts were made to sample patients who had a relapse without medication after obtaining consent from the patients and their guardians. Nevertheless, there were instances when steroids had to be administered once or twice as an emergency treatment before obtaining consent in severe cases of proteinuria and symptoms that led to emergency room visits. It is anticipated that obtaining prior consent from patients already diagnosed with SSNS to draw blood before initiating steroid therapy could enable more definitive results in future studies.

Moreover, despite numerous precautions to reduce potential bias during the phlebotomy process, unreported recent histories by patients or guardians may potentially influence the outcomes. Additionally, the age range set for blood sampling in this study was four years and above, which is typically when SSNS is diagnosed, and most patients sampled during the acute phase were pre-adolescent. On the other hand, chronic remission patients were often going through puberty or older, suggesting that age-related differences cannot be disregarded.

Finally, the study is limited by the lack of significant statistical differences in the changes of the *NLRC4* and *NAIP* genes upon podocyte stimulation and by the minimal differences between the groups stimulated with acute and chronic remission phase sera compared to those treated with acute phase serum plus DEX, with the exception of *IL1B*. While our approach of stimulating podocytes with serum followed by mRNA sequencing appears promising, the limited availability of serum from a larger patient cohort and the desire to explore longer and more varied exposure times remain practical challenges. Nevertheless, the identification of several inflammasome-related products with significant differences in podocytes is of meaningful implication.

V. CONCLUSION

In conclusion, this study provides significant evidence suggesting a crucial link between the activation of the NAIP/NLRC4 inflammasome and the pathogenesis of INS, particularly in SSNS, illuminating potential pathways for pathological alterations in podocytes. These findings extend our understanding of the immune mechanisms contributing to SSNS and present opportunities for targeted therapeutic intervention. However, considering the role of the NAIP/NLRC4 inflammasome in bacterial detection, further research is necessary to delineate the specific mechanisms in nephrotic syndrome and to develop therapeutic strategies that mitigate inflammatory responses.

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ABSTRACT IN KOREAN

소아 신증후군의 병인에서 인플라마좀의 역할

도입: 특발성 신증후군, 특히 스테로이드 반응성 신증후군은 잦은 단백뇨의 재발로 스테로이드를 반복적으로 투여하면서 발생하는 부작용으로 인해 고통을 받는 질환이다. 아직 원인을 잘 알지 못하는 이 질환의 기전을 인플라마좀과 연관하여 찾아보고자 하였다.

방법: 본 연구는 스테로이드 반응성 신증후군 환자의 급성기 및 만성 관해기 혈액을 분석하여 비교하고, 나머지 혈액에서 분리한 혈청으로 인간 족세포를 자극해 실험해 본 최초의 연구이다. 이 연구는 2016년부터 2023년까지 스테로이드 반응성 신증후군으로 진단된 4세 이상 18세 미만 환자들을 대상으로 연구 동의를 받아 진행하였다. 단백뇨가 많이 나오고 있는 환자를 급성기, 약물 치료 없이도 1년 이상 단백뇨 재발이 없는 환자를 만성 관해기로 분류한 뒤 진료를 위해 내원한 환자들을 대상으로 7.5cc 채혈하여 2.5cc는 RNA 시퀀싱 후 결과 데이터를 분석하고 여러 가지 방법으로 통계적 모델링을 진행하였다. 나머지 5cc는 채혈 즉시 혈청을 원심 분리하여 영하 140℃ 액체질소에 보관한 뒤 인간 족세포 실험에 사용하였다. 족세포는 배양 후 운반체(vehicle)로 자극한 그룹, 급성기 혈청으로 자극한 그룹, 급성기 혈청과 덱사메타손으로 함께 자극한 그룹, 만성 관해기 혈청으로 자극한 그룹으로 나누어 동시에 실험하였다. 이후 족세포 내 엑틴(actin) 필라멘트를 염색 후 현미경으로 확인한 뒤 혈청 분석과 마찬가지로 자극한 족세포들을 RNA 시퀀싱 후 결과 데이터를 분석하고 여러 가지 방법으로 통계적 모델링을 진행하였다.

결과: 스테로이드 반응성 신증후군 환자의 급성기 및 만성 관해기 전혈을 비교 분석한 결과 인플라마좀과 관련이 있는 유전자 중 *NLRC4, NAIP, NFKB1, MAPK14* 유전자들이 통계적으로 유의하게 급성기에서 활성화되어 있었다. 두 그룹간 차이가 나는 차등 발현 유전자들(differentially expressed genes, DEGs)은 3,554개였고 유전자 온톨로지(gene ontology) 농축 분석에서 급성기에서 프로그램화된 세포사 및 세

포자멸사 과정, 세포 생물합성 과정 및 세포 내 신호전달 MAPK 경로의 발현이 증가된 것이 확인되었다. 족세포를 환자 혈청으로 자극하는 실험에서는 급성기 혈청으로 자극된 세포는 다른 그룹에 비해 엑틴 필라멘트 감소를 보였고, 급성기 혈청과 덱사메타손을 동시에 처리한 세포는 일부 엑틴 필라멘트 회복이 나타났다. 또한, 급성기 혈청으로만 자극한 족세포 군에 비해 급성기 혈청과 덱사메타손을 동시에 처리한 족세포 군에서 *CASP3, IL1B, NFKB1* 유전자 활성도가 통계적으로 유의하게 낮은 것으로 관찰되었다. 두 그룹 간에는 유전자 온톨로지 농축 분석에서 2,153개의 DEGs 차이가 확인되었는데, 이 중에서 79개가 PodNet 데이터베이스와 겹치는 것으로 나타났다. 급성기 혈청으로 자극된 족세포 군에서, 급성기 혈청과 덱사메타손을 함께 처리한 세포 군에 비해 발현이 증가된 DEGs는 세포골격 조직 및 초분자 섬유 조직 관련 경로와 관계가 있었던 반면, 발현이 감소된 DEGs는 프로그램된 세포 사멸, 세포자멸사 및 대사 과정과 같은 경로와 관련이 있었다.

결론: 이 연구는 NLRP3와는 다른 특징을 가지고 있는 NAIP/NLRC4 인플라마좀이 스테로이드 반응성 신증후군의 병인과 중요한 연관성이 있음을 제시한다. NAIP/NLRC4 인플라마좀과 관련된 염증 유발 사이토카인들은 세균 감염이나 면역반응의 이상으로 인한 단백뇨의 발생 및 악화와 잠재적으로 연결될 수 있으며, 이들이 염증 유발 신호와 항염증 신호 간의 균형을 깨면 족세포의 병리학적 변화로 이어질 수 있다. 향후에는 이러한 물질들의 신증후군에서의 정확한 기능과 상호 작용을 명확히 하고 치료 전략을 세우는 추가적인 연구가 필요하다.

핵심되는 말: 신증후군, 특발성 신증후군, 스테로이드 반응성 신증후군, 인플라마좀, 인간 족세포