WEP Warwick Evans Publishing

International Journal of Public Health and Medical Research

ISSN: 3006-7707 (Print), ISSN: 3006-3752 (Online) | Volume 1, Number 2, Year 2024
DOI: https://doi.org/10.62051/ijphmr.v1n2.10
Journal homepage: https://wepub.org/index.php/IJPHMR/index

Immunologically Mediated Mechanisms in Renal Disease

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ABSTRACT

The immunologically mediated mechanisms within renal diseases represent a complex biological process. This study primarily focuses on analyzing the roles and mechanisms of immune cells, specifically macrophages, T cells, and B cells, in renal pathology. Utilizing a meticulous approach that combines bioinformatics analysis with in vitro experiments, this paper reveals the critical role of m6A RNA methylation modifications in regulating immune cell functions, promoting apoptosis, and inflammatory responses. The results suggest that the interactions among immune cells and their impact on renal health are of significant biological importance, and that the regulation of m6A methylation modifications presents new potential targets for renal disease treatment. However, as the study relies on in vitro experiment results, the findings necessitate further validation through additional animal experiments and clinical trials. Future research should delve deeper into the specific roles and interactions of different immune cells in various renal diseases to develop more effective treatment strategies.

KEYWORDS

Renal Disease; Immunologically Mediated Mechanisms; m6A RNA Methylation; Immune Cells; Therapeutic Targets.

1. INTRODUCTION

In many developed countries, renal disease has become the most common cause of end-stage renal disease, associated with oxidative stress, inflammatory responses, abnormalities in glucose and lipid metabolism, hemodynamic changes, and genetic factors. These conditions lead to diffuse glomerular mesangial expansion and sclerosis under hyperglycemic conditions, thickening of the glomerular basement membrane, reduction of podocytes, fusion or disappearance of foot processes, interstitial fibrosis, and tubular atrophy. The pathogenesis of renal disease is complex, and current treatment methods are limited in effectiveness [1, 2]. Therefore, further exploration of the molecular mechanisms of renal disease will aid in identifying new potential therapeutic targets, providing new approaches for early diagnosis and treatment of renal disease [3, 4].

1.1. Immune Regulatory Mechanisms in Renal Disease

Immune cells play a crucial role in both health and disease states [5]. They are key executors of the immune system, responsible for recognizing and eliminating pathogens, regulating inflammatory responses, and maintaining tissue homeostasis. In renal disease, studying the role of immune cells is particularly important, as the kidneys are major metabolic and excretory organs in the human body, and damage to them can lead to complex systemic diseases [6]. Understanding the role of immune cells in renal disease not only helps reveal the mechanisms of the disease but also provides a theoretical basis for developing new treatment methods [7].

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Macrophages: As key cells of the innate immune system, macrophages play important roles in clearing infections, regulating inflammatory responses, and promoting tissue repair. In the kidneys, macrophages phagocytize dead or damaged cells, release inflammatory factors, and promote fibrosis, affecting the health status and pathological process of the kidneys.

T cells: T cells are central to the adaptive immune response and can be divided into helper T cells, suppressor T cells, and cytotoxic T cells, depending on their function. In renal disease, T cells regulate immune responses by recognizing specific antigens, influencing the development of inflammation and autoimmune reactions.

B cells: B cells primarily mediate immune responses through the production of antibodies. Antibodies can bind to pathogens, promoting their clearance, and may also participate in the formation of pathological immune complexes, triggering renal inflammation and damage.

Immune cells may have a dual role in renal disease, protecting the kidneys from infection and damage, but also potentially causing inflammation and damage to renal tissue through excessive or abnormal responses. For example, the activation of macrophages and T cells in diabetic nephropathy and autoimmune nephritis can exacerbate tissue inflammation, promoting disease progression. In the inflammatory process of renal disease, various immune cells not only act alone but also interact with each other to synergistically affect disease development. Macrophages release cytokines that activate neighboring T cells and B cells, and the activation of T cells and B cells, in turn, enhances the inflammatory response of macrophages. Moreover, the interactions between T cells and B cells are also crucial, especially in regulating autoimmune responses. Helper T cells can promote the maturation of B cells and the production of antibodies, which may be involved in the formation of renal lesions, such as immune complexes formed in systemic lupus erythematosus nephritis, depositing in the glomeruli and causing inflammation and tissue damage.

1.2. Disease-Specific Mechanisms

In various renal diseases, antibodies and immune complexes not only act as initiators of the pathological process but also affect the structure and function of the kidneys through multiple mechanisms [8]. They activate the complement system, which not only attracts a large number of inflammatory cells such as macrophages and neutrophils but also prompts these cells to release inflammatory mediators and enzymes [9]. These factors collectively damage the kidney's structure and impair its function [10].

RNA methylation refers to the chemical modification where methyl groups are selectively added to methyladenine in RNA, catalyzed by methyltransferases, primarily in the form of m6A methylation. This modification is reversible and involves the cooperation of methylation transferases (Writers), demethylases (Erasers), and methylation-reading proteins (Readers). m6A methylation mainly occurs on adenosine, specifically at the N6 position of the adenine base, and is enriched near stop codons and in the 3' untranslated regions (UTRs), making it the most common, conserved, and abundant internal modification in RNA. The "Writers" introduce a methyl group to the adenosine base in the RRACH sequence (R represents purine A or G, H represents adenine, cytosine, or uracil - A, C, or U) near the stop codon, with the highest abundance at the transcription start site (TSS) and 3'-UTR. The "Erasers," primarily FTO and ALKBH5, mediate demethylation, making m6A a dynamic and reversible process. The "Readers" are a group of RNA-binding proteins that recognize m6A methylation and execute corresponding functions. Both mRNA and lncRNA abundantly feature m6A modifications, which can accelerate the processing time of mRNA precursors, and enhance the transport and nuclear export speed of mRNA within cells.

Cell pyroptosis and immune inflammation are significant modes of renal cell death; thus, unveiling the mechanisms of m6A methylation in these processes holds important clinical significance for the prevention and treatment of renal diseases. Accordingly, this study first employs bioinformatics analysis methods to identify genes related to m6A methylation in renal disease and their regulatory

relationships with cell pyroptosis and inflammation. It explores the involved signaling pathways and biological processes and analyzes their immune regulatory mechanisms. Subsequently, in vitro experiments validate the results of the bioinformatics analysis, providing new insights into the development of diabetic renal disease.

2. MATERIALS AND METHODS

2.1. Material Preparation

Human podocyte cell line (HPC) was purchased from Shanghai Qincheng Biotechnology Co., Ltd., with product number QC822. HPCs were cultured in 89% McCoy’s 5a medium containing 15 mmol/L D-glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture. The cells were maintained in a constant temperature incubator at 37 ℃ with 5% CO_2 and were continuously cultured without the use of double antibiotics during and after transfection.

Human kidney cortex proximal tubular epithelial cells (HK-2) were acquired from Puno Sai Life Science Technology Co., Ltd., product number CL-0109. The cells were cultured in Minimum Essential Medium (MEM) with a D-glucose concentration of 5.5 mmol/L, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture. The cells were maintained in a constant temperature incubator at 37 ℃ with 5% CO_2 and were continuously cultured without the use of double antibiotics during and after transfection.

2.2. Experimental Methods

Prior to the experiment, the necessary items were placed in a laminar flow cabinet and exposed to ultraviolet light for 30 minutes (sterile operations must be strictly adhered to during cell experiments). According to the manual for HPC cells, the cryopreserved HPC cells were quickly retrieved from liquid nitrogen storage, rapidly thawed in a 37 ℃ water bath by shaking, ensuring the cells dissolved quickly. Once completely dissolved, the HPCs were immediately diluted with complete culture medium (10% FBS, 89% McCoy's 5a, 1% double antibiotic) and the HK-2 cells were similarly diluted using complete culture medium (10% FBS, MEM with NEAA, 1% double antibiotic). The cells were then centrifuged at 4 ℃ at 1000 rpm/min, the supernatant was discarded, and 3 ml of complete culture medium was added to the centrifuge tube. The cells were resuspended by repeated pipetting, then transferred to a T25 culture flask, shaken to mix, and observed under a microscope to assess cell morphology. The T25 culture flasks were then placed in a 37 ℃, 5% CO2 incubator, and the cell morphology was periodically observed, changing the medium as necessary.

Cell growth was monitored by removing the culture dish from the incubator and observing the cell morphology and density under a microscope. When the cell density reached approximately 80-90%, the cells were suitable for passaging. The original medium was discarded, and the cells were washed twice with 2 ml of precooled PBS. Then, 1 ml of 0.25% trypsin was added to the culture flask for cell digestion, and placed back in the incubator for about 1 minute. Under the microscope, if cells appeared round and began to float, digestion was deemed successful. Immediately, 2 ml of medium was added to the flask to stop the digestion, and the cells were gently pipetted to ensure even mixing. The cell suspension was then transferred to a sterile centrifuge tube and centrifuged at 4 ℃, 1200 rpm/min for 5 minutes. After centrifugation, the supernatant was discarded, and 3 ml of culture medium was added to resuspend the cells, which were then transferred to a new T25 culture flask and placed back into the incubator to adhere. This study used generations 3 to 8 of HPC and HK-2 cells.

Prior to cryopreservation, experimental items were placed in a laminar flow cabinet and irradiated with ultraviolet light for 30 minutes. Cells with good morphology and in the logarithmic growth phase were selected for cryopreservation. The day before freezing, the original culture medium was replaced with fresh medium to maintain cells in optimal condition. Following the cell passaging methods

described above, 1 ml of cryopreservation solution was used to gently resuspend the cell pellet. The cells were then transferred to a cryotube, the cap was tightened, sealed with sealing film, and labeled with the cell name, required medium, cell generation, freezing time, and freezer's information for future reference. The cells were then placed in a 4 ℃ refrigerator for 30 minutes, transferred to a -20 ℃ freezer for 2 hours, and finally placed in an -80 ℃ freezer overnight. The next day, they were moved to a liquid nitrogen tank for long-term storage.

This study involved transfecting small interfering RNA (siRNA) and plasmid overexpression to knock down and overexpress FTO in cells, respectively. Cells were transiently transfected with si-FTO/FTO OE and si-NC/vector in HPC and HK-2 cells using INVI DNA RNA Transfection Reagent (IV1216150, Invigentech, USA). The transfection efficiency was verified by real-time quantitative PCR. The siRNA for knocking down FTO and the plasmid for FTO overexpression, as well as the negative controls, were constructed by Shanghai Jikai Gene Company. 24 hours after transfection of si-FTO/FTO OE and si-NC/vector in HPC/HK-2 cells, the cells were stimulated with high glucose (30mM) and lipopolysaccharide (10 mg/L) for another 24 hours. Protein expression and mRNA levels in the cells were then measured by Western blot and real-time quantitative PCR, respectively.

HPC in the logarithmic growth phase were seeded into a 96-well plate at a density of 10,000 cells per well and cultured for 24 hours to allow for cell adhesion. A gradient of glucose concentrations (30 mM, 60 mM, 90 mM, 120 mM) was established and maintained for 24 hours. To facilitate the calculation of absorbance results, blank wells were also prepared. Using a pipette, 10 μL of CCK-8 reagent was added to each well, taking care to avoid bubble formation that could affect the accuracy of the measurements. After adding the reagent, the 96-well plate was protected from light and incubated for an additional 2 hours in the incubator. The absorbance at 450 nm was then measured using an automatic microplate reader, and cell viability was calculated according to the manufacturer's instructions as follows: Cell Viability = (OD of the treated group - OD of the blank group) / (OD of the control group - OD of the blank group).

2.2.1. Real-Time Quantitative Fluorescence

Place the samples on ice, first discard the original culture medium and wash the cells twice with PBS. Then add 1 ml of TRIzol reagent to each well, place on ice for 5 minutes, and mix thoroughly by pipetting. Transfer the mixture to enzyme-free EP tubes and add 0.2 ml of chloroform to each tube. After vigorous shaking and vortexing, let it stand on ice for 10 minutes. Then centrifuge at 4 ℃ and 12,000 rpm for 10 minutes. Transfer 400-600 μl of the supernatant to a new enzyme-free EP tube, add an equal volume of isopropanol, and mix well by vortexing. Let it stand on ice for another 10 minutes, then continue to centrifuge under the same conditions for 10 minutes. Discard the supernatant and retain the white precipitate, wash the precipitate twice with 1 ml of 75% ethanol, and after each wash, centrifuge at 4 ℃ and 12,000 rpm for 5 minutes. Finally, open the EP tube and allow the residual water and ethanol to evaporate in a dry condition for about 2 minutes. Dissolve the RNA in DEPC-treated water based on the RNA quantity and store at -80 ℃ for later use.

Measure the concentration and purity of the total RNA extracted from each treatment group using a microplate reader. Dilute the total RNA with DEPC water to a concentration within the range of 10-100 ng/μl. Use the RNA Reverse Transcription Kit (FastKing cDNA First Strand Synthesis Kit) (see Table 1). Perform all operations on ice and protect light-sensitive reagents from exposure. Before use, thoroughly vortex each component of the kit to mix, and briefly centrifuge to collect any liquid from the tube walls. Prepare the genomic DNA removal mix, mix thoroughly, and briefly centrifuge, then incubate in a gradient PCR machine at 42 ℃ for 3 minutes, then place on ice. Next, prepare the reverse transcription reaction mix, add the reverse transcription Mix to the gDNA removal reaction, and mix thoroughly. Place in the gradient PCR machine at 42 ℃ for 15 minutes, then at 95 ℃ for 3 minutes before cooling on ice. The resulting cDNA can be used for subsequent experiments or stored at -20 ℃.

Table 1. Reverse Transcription Kit Sample Preparation Table

Composition	Volume
2×Talent qPCR PreMix(SYBR Green)	1.25 ml
50×ROX Reference Dye	250μI
RNase-Free ddH20	1 ml

2.2.2. Western Blotting

Remove cells from the incubator and place them on ice. Wash the cells once with precooled sterile 1×PBS. To each well, add 200 μl of cell lysis buffer (200 μl high-efficiency RIPA buffer; 2 μl phosphatase inhibitor; 2 μl PMSF), and lyse the cells on ice for 30 minutes. Using a cell scraper, scrape the cells in one direction into a 1.5 ml EP tube. Centrifuge at 4 ℃ and 12,000 rpm for 10 minutes. Transfer the supernatant to a new EP tube and store at -80 ℃ for later use.

According to the instructions of the Bicinchoninic Acid (BCA) Protein Assay Kit by Beyotime, working solution was prepared with a ratio of Reagent A to Reagent B of 50:1. For the preparation of protein standards using a 96-well plate: First, different volumes of a 0.5 mg/ml protein standard—0, 1, 2, 4, 8, 12, 16, 20 μl—were added to the wells, followed by addition of pure water in volumes of 20, 19, 18, 16, 14, 12, 8, 4, 0 μl respectively to reach a final volume of 20 μl. This corresponds to protein standard concentrations of 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml, respectively, used for plotting the protein standard curve. For experimental samples, 2 μl of cell lysate was added to the remaining wells, with four replicates per treatment to minimize intra-group variability. Each well then received 200 μl of BCA working solution, and the plate was incubated in a 60 ℃ metal bath in the dark for 30 minutes. Absorbance at 562 nm was measured using a spectrophotometer. A standard curve was created in Excel plotting OD values against protein concentrations. Protein concentrations of the experimental samples were calculated by entering their OD values into the standard curve equation. Finally, a quarter volume of 5x SDS sample buffer was added to the samples, mixed, and heated in a 100 ℃ water bath for 8-10 minutes before storage at -20 ℃ until further analysis.

The prepared protein samples were removed and placed on ice. Electrophoresis equipment was set up using a 4-12% FuturePAGE™ precast gel, and the electrophoresis buffer was prepared according to the MOPS-SDS Running Buffer instructions. After removing the comb from the gel, 4 μl of marker and the protein samples from each treatment group were loaded into the wells. Electrophoresis was carried out at a constant voltage of 160V for 40 minutes, adjusting the time according to the molecular weights of the proteins. After electrophoresis, the gel was transferred to a membrane using a constant current. The gel was placed in transfer buffer and a PVDF membrane was cut to the appropriate size and activated in methanol for about 5 minutes. Sponges and filter papers of the right size were prepared and placed in the transfer tank, surrounded by ice packs to prevent overheating. The transfer was carried out at a constant current of 200 mA for 60-120 minutes, adjusting the time according to the protein molecular weights.

After transfer, the PVDF membrane was soaked in blocking solution, specifically 4% skim milk, and agitated gently on a shaker at room temperature for approximately an hour or blocked quickly with a fast blocking solution for 15-20 minutes. After blocking, the membrane was cut according to the marker indications to isolate the target protein bands. The membrane was then washed three times for 5 minutes each with TBST to remove any remaining blocking solution. The strips were placed in the primary antibody solution, agitated gently on a shaker for 3 hours or incubated overnight in a 4 ℃ refrigerator. The following day, the strips were washed three times for 10 minutes each with TBST. The secondary antibody was prepared in 4% skim milk and chosen based on the primary antibody host species; it was then incubated with the membrane at room temperature on a shaker for at least an hour. After incubation, the strips were washed quickly three times for 10 minutes each with TBST, and prepared for exposure and development.

2.2.3. Immunoprecipitation

After removing the culture medium, wash the cells once with precooled 1×PBS. Add precooled lysis buffer (containing 1 mM PMSF final concentration) to each well and lyse on ice for 5 minutes. Scrape the cells, perform a brief micro-centrifugation, then sonicate three times for 15 seconds each on ice. Centrifuge at 4 ℃ and 12,000 rpm for 10 minutes. Transfer the supernatant to an EP tube. The lysate can be stored at -80 ℃.

Take 200 μl of the supernatant for protein quantification to standardize the concentration for the input group. In a new EP tube, add 500 μl of cell lysate (containing 200-1000 μg total protein), which may be pre-cleared lysate. Add 1-5 μg of monoclonal/polyclonal antibody specific to the target protein. Use a nonspecific homologous antibody as a control. Mix gently at 4 ℃ overnight.

After overnight incubation with the antibody, add 5 μl of Protein A and 5 μl of Protein G, and mix gently at 4 ℃ for 1-3 hours or overnight. The next day, centrifuge at 12,000 g for 1 minute and retain the pellet. Wash the pellet with 0.5 ml of 1×Wash buffer, centrifuge at 12,000 g for 1 minute, and retain the pellet. Repeat the washing of the pellet with another 0.5 ml of 1×Wash buffer, followed by centrifugation at 12,000 g for 1 minute, and perform this washing step three times in total.

Resuspend the pellet in 20-40 μl of 1×SDS sample buffer. Vortex and mix well, then briefly spin down for 30 seconds to ensure any beads and liquid attached to the tube walls are collected at the bottom of the tube. Heat the samples at 95-100 ℃ for 2-5 minutes. Then, centrifuge instantaneously at 14,000 g for 1 minute and collect 15-30 μl of the supernatant. Load the sample onto an SDS-PAGE gel for protein blotting analysis, following the same steps as described previously.

2.2.4. Total RNA m6A Methylation Level in Cells

First, prepare 1×Wash Buffer by adding 18 ml of 10×Wash Buffer to 122 ml distilled water to make 1×WB. Then dilute CA solution at a 1:1000 ratio using diluted 1×WB, dilute DA at a 1:2000 ratio, and dilute ES at a 1:5000 ratio, preparing all solutions to their working concentrations for use.

Remove the required number of 8-strip wells from the kit. Add 80 μl of Binding Solution (BS) to each well, followed by 2 μl of Negative Control (NC), 2 μl of diluted Positive Control (PC) solution, and 2 μl of RNA sample containing 200 ng (the RNA amount per reaction ranges from 100 ng to 300 ng). Mix the solutions gently by tilting the plate from side to side to ensure the liquid evenly covers the bottom of the wells, and seal the plate with sealing film. Secure the plate with a plate sealing strip and incubate in a 37 ℃ metal bath for 90 minutes.

After incubation, remove the BS from each well. Add 150 μl of diluted Wash Buffer (WB) to each well using a pipette, and repeat the wash three times for 5 minutes each. Add 50 μl of Capture Antibody (CA) solution and place in a 37 ℃ incubator for 1 hour. After removing the CA solution, wash three times with WB for 5 minutes each. Add 150 μl of Detection Antibody (DA) solution and incubate for 30 minutes, then discard the DA solution and wash four times with WB for 15 minutes each. Add 50 μl of Enzyme Solution (ES) and incubate at room temperature for 30 minutes, followed by three washes with WB for 5 minutes each.

Add 100 μl of Developer Solution (DS) and incubate in the dark for 10 minutes until the color changes to bright blue. Stop the reaction with Stop Solution (SS), which will change the solution color to yellow. Measure the absorbance at 450 nm using a spectrophotometer within 15 minutes. Calculate the percentage of m6A using the formula:

$$m6A\% = [(Sample\ OD - NC\ OD)/S]/[(PC\ OD - NC\ OD/P] * 100\% \quad (1)$$

Here, S is the amount of RNA in the sample in ng, and P is the amount of RNA in the positive control in ng.

2.2.5. Annexin V-APC/PI Dual Staining for Cell Apoptosis

Seed HPC and HK-2 cells in logarithmic growth phase in a 6-well plate, establishing blank controls and treatment groups. After 24 hours of treatment, digest the cells with 0.25% trypsin without EDTA (carefully control the digestion time as prolonged digestion affects the binding of phosphatidylserine on the cell membrane to Annexin V). Terminate the digestion promptly and collect the cells into a centrifuge tube. Centrifuge at 1200 rpm for 3 minutes, collect the cell pellet, and resuspend in precooled 1×PBS. Wash the cells by centrifugation at 1200 rpm for 3 minutes. Dilute 10×Binding Buffer with DEPC water to make 1×Binding Buffer, and resuspend the cells in 500 μl of 1×Binding Buffer per group.

For each group, add 5 μl of Annexin V-APC and Propidium Iodide (PI) and mix well. Incubate at room temperature in the dark for 15-20 minutes. The negative control consists of normal cells without the addition of Annexin V-APC and PI. Positive control one receives only 5 μl of Sul Annexin V-APC, and positive control two receives 5 μl of PI. Place the prepared cell samples in a flow cytometer for analysis to primarily observe the proportion and number of cells in different states, to assess cells in early apoptosis, late apoptosis, and death.

- The upper left quadrant (Annexin V-/PI+) may represent necrotic cells or cell debris lacking intact cell membranes, or cells that died due to other reasons.

- The lower left quadrant represents normal (live) cells (Annexin V-/PI-).

- The upper right quadrant indicates late apoptotic cells (Annexin V+/PI+).

- The lower right quadrant shows early apoptotic cells (Annexin V+/PI-).

2.2.6. Cell Migration Scratch Assay

After digesting and preparing the cell suspension, take 10 μl of the cell suspension for cell counting. Use a low-power microscope to count the cells in the four large squares at the corners, applying the counting rule of "count left, not right; count top, not bottom". Calculate the cell number using the formula: (total cells in four corners / 4) ×10^4 cells/ml. Take a 12-well plate, draw parallel lines on the back of the 12-well plate, adjust the cell suspension density, and place in a 37 ℃, 5% CO_2 incubator.

2.2.7. Statistical Analysis

Ingenuity Pathway Analysis (IPA) software, a cloud-based bioinformatics platform with a graphical interface, is used to validate the regulatory relationships between m6A regulatory factors and pyroptosis genes. IPA enables the analysis, integration, and understanding of omics data from a biological pathway perspective and is suitable for large-scale data analysis in transcriptomics, proteomics, and metabolomics, as well as for smaller-scale experiments that generate lists of genes and chemicals. The main outputs of omics data analysis include canonical pathways, upstream transcriptional regulators, downstream regulatory effects, diseases and functions, and networks of molecular interactions.

Statistical analysis of the data is conducted using SPSS and GraphPad Prism software. For variables that follow a normal distribution, the Independent Samples t-test is employed for comparisons between two groups. For variables that do not follow a normal distribution, the Mann-Whitney U test is used. Comparisons among multiple groups are performed using One-Way Analysis of Variance (ANOVA), and for non-normally distributed quantitative data, the Kruskal-Wallis test is applied. Correlation analyses are conducted using the Spearman correlation coefficient. A p-value of <0.05 is considered statistically significant. Image processing is carried out using ImageJ software.

3. RESULTS

3.1. m6A Methylation Levels in HPC Cells

The levels of m6A modification in different treatment groups of HPC cells were measured following the protocol of the m6A RNA Methylation Quantification Kit. The experimental groups included the normal control group (CON), high glucose group (HG), high glucose + lipopolysaccharide group (HG+LPS), high glucose + lipopolysaccharide + transfection control group (si NC), high glucose + lipopolysaccharide + FTO knockdown group (siFTO), high glucose + lipopolysaccharide + empty vector group (Vector), and high glucose + lipopolysaccharide + FTO overexpression group (FTO OE). The results showed no significant differences in the levels of m6A expression between the various treatment groups. However, there was a trend of increased overall m6A levels in the CON group compared to the HG+LPS group; the HG+LPS group also showed a rising trend in m6A levels compared to the siFTO group, while m6A levels tended to decrease when compared to the FTO OE group (as shown in Figure 1).

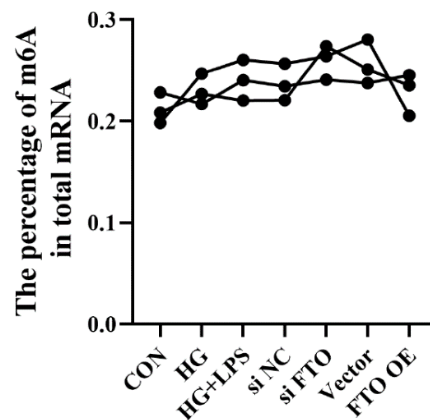

Figure 1. Overall m6A Methylation Levels in HPC Cells

3.2. Concentrations of High Glucose and Lipopolysaccharide Inducing Damage in HPC Cells

CCK8 assay results indicate that at a glucose concentration of 30 mM and after 24 hours of treatment, the survival rate of HPC cells was 87%, which is lower than that of the normal control group ($P<0.05$) (as shown in Figure 2). Therefore, this study chose a glucose concentration of 30 mM and a treatment duration of 24 hours as the high glucose (HG) model to induce podocyte damage.

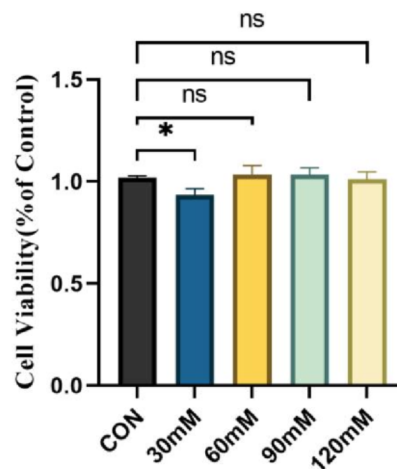

Figure 2. Survival Rates of HPC Cells after Stimulation with Different Concentrations of High Glucose

On the basis of HG-induced damage, a gradient of different lipopolysaccharide (LPS) concentrations (10 mg/L, 20 mg/L, 40 mg/L, 60 mg/L) was established and treated for 24 hours. Western Blot results (as shown in Figure 3) show that compared to the blank control group, the HG treatment group exhibited decreased levels of Nephrin and Podocin, while inflammatory factors IL-1 and IL-18 were upregulated, indicating that a glucose concentration of 30 mM is damaging to HPC cells. Further, upon adding different gradients of LPS on the basis of HG damage, it was found that compared to the blank control group, the HG 30 mM + LPS 10 mg/L group showed further reduction in Nephrin and Podocin (P<0.01) and significant upregulation of IL-1β and IL-18 (P<0.001). These results suggest that HG+LPS can further damage podocytes and enhance the inflammatory response.

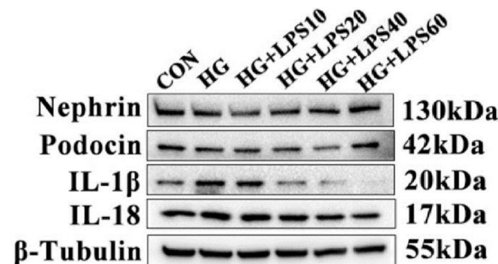

Figure 3. Western Blot Analysis of Podocyte Marker Proteins and Inflammation-Related Proteins Expressed under High Glucose Conditions with Different Concentrations of Lipopolysaccharide Induced;**

4. CONCLUSION

This study thoroughly examined the regulatory mechanisms of immune cells in renal diseases, elucidating the complex role of the immune system in renal pathology, particularly the dual role of macrophages, T cells, and B cells in the progression of the disease. The results demonstrate that m6A RNA methylation modifications play a crucial role in regulating immune cell functions and influencing cell apoptosis and inflammatory responses, highlighting potential therapeutic strategies through the modulation of m6A methylation. The findings of this paper are significant for understanding the immunoregulatory mechanisms in kidney diseases. Firstly, it revealed the regulatory role of m6A methylation in kidney diseases, offering possibilities for the discovery of new therapeutic targets. Secondly, the relationship between immune cells and kidney diseases was validated through in vitro experiments, enhancing the understanding of kidney diseases at the molecular level. However, there are some limitations in the study. For instance, the research relies heavily on in vitro results and lacks sufficient animal models and clinical data to support the universality of the findings. Moreover, the specific mechanisms of interaction between immune cells are not fully understood and require further investigation.

Future research should focus on the following areas: first, further validation of the role of immune cells in kidney diseases through animal models and clinical trials; second, a deeper exploration of the specific impacts of different immune cells and their interactions on renal pathology; third, exploration of the potential roles of m6A methylation modifications in other types of kidney diseases. Through these studies, more effective treatment methods are hoped to be developed to address this complex disease.

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