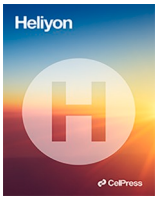
Heliyon 10 (2024) e37866

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Exosomes secreted by podocytes regulate the differentiation of Th17/Treg cells in idiopathic nephrotic syndrome

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ARTICLE INFO

Keywords:
Nephrotic syndrome
Exosomes
Podocyte
Th17 cells
Treg cells

ABSTRACT

Background: Previous studies have demonstrated that immune cells release exosomes, which act as antigen-presenting vesicles to activate T cells. In our previous study, we discovered that podocytes, a type of kidney cell, can also exhibit antigen-presenting functions to naïve $CD4^+$ T cells in idiopathic nephrotic syndrome (INS). Building upon these findings, the objective of this study was to investigate whether podocytes can regulate the balance between Th17 and Treg cells through the release of exosomes.

Methods: We co-cultured naïve $CD4^+$ T cells with LPS-treated bone marrow dendritic cells (LPS-BMDC), LPS-treated mouse podocyte clone 5 (LPS-MPC-5), and exosomes derived from LPS-MPC-5 (LPS-EXO). As a control group, naïve $CD4^+$ T cells were cultured with exosomes from untreated MPC-5 (EXO). After 48 h, we analyzed the percentages of Th17 and Treg cells using flow cytometry, measured the concentrations of IL-17A, IL-10, and IL-4 were using ELISA, and examined the expressions of IL-17a, IL-10, RORC, and FOXP3 using RT-qPCR.

Results: We confirmed the presence of exosomes derived from podocytes based on their morphology, size distribution, concentrations, and the levels of exosomes-specific markers. The percentage of Th17 and Treg cells in the LPS-EXO group was significantly higher than that in the control groups, but lower than in the LPS-MPC-5 group. Furthermore, the ratio of Th17/Treg was relatively higher in the LPS-EXO group compared to the LPS-MPC-5 group.

Conclusion: This study indicated further insights into the role of exosomes released from LPS-treated podocytes in regulating the balance between Th17 and Treg cells in INS.

1. Introduction

Idiopathic nephrotic syndrome (INS) is the most common form of nephrosis in children, characterized by significant proteinuria, hypoalbuminemia, and edema [1]. Despite extensive research over several decades, the pathogenesis of INS remains incompletely understood. Indirect evidence from earlier studies suggests a close association between T cell dysfunction or dysregulation and INS. For example, patients with INS may benefit from the use of calcineurin inhibitors such as tacrolimus and cyclosporin [2]. In addition, Abatacept, a fusion protein of CTLA-4-Ig that blocks $CD28^-CD80/86$ pathway of T cells, has been shown to induce remission of proteinuria in Adriamycin-induced nephropathy (AIN) rats [3]. Liu et al. found that patients with NS had a higher Th17/Treg ratio, which was positively correlated with 24 h proteinuria, compared to healthy controls [4]. In our previous study, we demonstrated that

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https://doi.org/10.1016/j.heliyon.2024.e37866
Received 14 January 2024; Received in revised form 10 September 2024; Accepted 11 September 2024
Available online 12 September 2024

Abatacept could modulate IL-17/Treg levels and improve proteinuria in AIN rats [3]. These results suggest an involvement of Th17/Treg imbalance in INS. However, the specific factor that disrupts the balance between Th17 and Treg cells is still unknown.

Podocytes, the final defense against protein loss in the glomerular filtration barrier, utilize their negatively charged apical surface to restrict the passage of negatively charged proteins, with foot processes connecting to the glomerular basement membrane and regulating barrier permeability through actin bundles [5,6]. In addition, podocytes also possess various immune cell features, such as antigen presentation, expression of co-stimulatory molecules (such as CD80), and activation of T lymphocytes [7,8]. Within the immune microenvironment, both innate and adaptive immune components induce podocyte injury. Subsequently, the injured podocytes can engage as immune cells with inflammatory cells (such as macrophages, neutrophils and monocytes) and T cells [9].In our *in vitro* study, we found that podocytes treated with lipopolysaccharide (LPS) could enhance differentiation of T lymphocytes into Th17/Treg cells, even in the absence of upregulated CD80 and MHC-II expression [10]. This finding suggested the involvement of other factors, besides CD80 and MHC-II, play a role in T cells differentiation [10].

Extracellular vesicles (EVs) are lipid bilayer-enclosed particles that transport various compounds, including proteins, lipids, nucleic acids, and metabolites. These EVs serve as a cell-to-cell communication mechanism, facilitating the transport of signals and influencing various processes and reactions in recipient cells [11,12]. Depending on their size, EVs can be classified as exosomes, microvesicles, apoptotic bodies and oncosomes [13].Recently, there has been growing interest in studying EVs generated from podocytes in the field of nephrosis. Elevated levels of podocyte-derived EVs in urine have been observed in individuals with metabolic syndrome-related kidney disease [14], renovascular hypertension [15], and preeclampsia [16], indicating a direct association with renal dysfunction. These finding suggest that podocyte-EVs could serve as early markers of kidney disease and potential therapeutic targets. However, limited studies have been conducted on the pathogenic mechanism of EVs. *In vitro* study has shown that EVs from podocytes treated with puromycin amino nucleoside or high glucose might induce apoptosis in tubular epithelial cells, potentially mediated by the transport of microRNAs via EVs [17,18].

Because podocytes share many characteristics with antigen-presenting cells [10], and immune cells release antigen-presenting vesicles to activate T cells [19,20], it is worth investigating whether podocytes can influence balance of Th17 and Treg cells through the release of EVs such as exosomes. In this study, we cultured naïve $CD4^+$ T cells with exosomes extracted from LPS-treated MPC-5 cells to determine the role of exosomes in modulating the balance between Th17 and Treg cells.

2. Materials and methods

2.1. Cells culture and treatment

Naïve $CD4^+$T cells and bone marrow dendritic cells (BMDCs) were collected from male C57BL/6 mice (6–8 weeks old) following previously described protocols [10]. The mouse podocyte clone 5 (MPC-5) cells (Icell, Shanghai, China) were cultured in DMEM medium at 37 °C in a humidified incubator with 5 % CO2. The MPC-5 cells were stimulated by LPS (eBioscience, USA) for 6 h, and the harvested BMDCs were also treated with 25 μl/ml LPS for 6 h. $CD4^+$ T cells were cultured in RPMI-1640 medium (Sigma, Germany) supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotics (penicillin/streptomycin) at 37 °C in a 5 % CO2 humidified incubator. Exosomes (20μg/well) derived from MPC-5 cells with or without LPS stimulation were used to culture with $CD4^+$ T cells for 48 h. LPS-treated BMDC and MPC-5 were separately co-cultured respectively with $CD4^+$ T cells for 48 h at a 1:1 ratio.

2.2. Exosomes isolation and identification

The exosomes were isolated from the supernatants of MPC-5 cell cultures. The supernatants were first centrifuged at 400 g for 5 min to remove cells and debris. Then, the supernatants were further centrifuged at 3000 g for 30 min to remove larger debris. The resulting supernatant was filtered using a 0.22 μm filter, and the filtrate was ultracentrifuged at 100,000 g for 120 min (Ultracentrifuge; Beckman Instruments, USA). The resulting pellet was diluted with 50ul PBS and stored at −80 °C for further experiments.

The morphology of exosomes was examined using transmission electron microscopy (TEM). The samples were placed on a formvar/carbon-coated copper grid for 5 min and then stained with 4 % uranyl acetate for 10 min. The exosomes on the grid were visualized using a transmission electron microscope (JEM-1400 PLUS; JEOL) after air-drying at room temperature. Nanoparticle tracking analysis (NTA) was performed to evaluate the size distribution and concentrations of exosomes using the ZetaView PMX 110 instrument (Particle Metrix, Meerbusch, Germany) and the corresponding software ZetaView 8.04.02. The isolated exosomes were appropriately diluted with 1 X PBS buffer for the measurement of particle size and concentration. The expression of exosomes-specific markers (CD6, CD63, and TSG101) were analyzed by Western blotting (WB).

2.3. Flow cytometry of immune cells

The specific monoclonal antibodies CD4-FITC, IL-17A-PE, CD25-PE-Cyanine7, FOXP3-APC for flow cytometry were obtained from eBioscience. For intracellular staining of IL-17A and FOXP3, cells were fixed in Intracellular Fixation buffer (eBioscience, USA) and permeabilized using Permeabilization Buffer (eBioscience, USA). The cells were then incubated with specific antibodies in the dark at room temperature for 30 min for surface antigens staining.

2.4. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol (Thermo, USA) and reverse-transcribed into cDNA using HiFiScript cDNA Synthesis Kit (Cwbiotech, China) following the manufacturer's recommendations. The expression of transcripts was examined using UltraSYBR Mixture (Cwbiotech, China) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reaction condition was as follows: heating to 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. The relative expression levels of mRNAs were evaluated by the $2^{-\Delta\Delta Cq}$ method. The primer pairs for RT-qPCR were designed using Primer Premier 5.0 software (Premier, CAN) (see Table 1 for primer sequences).

2.5. Western blotting analysis

Exosomes were quantified using the BCA protein kit (Abiowell, China) and subjected to western blotting (WB) to determine the protein levels of exosomal surface markers, following previously described methods [18]. Briefly, exosomes proteins were separated and transferred onto polyvinylidene fluoride membranes, and then were incubated with the following primary antibodies: anti-CD9 (1:1000; rabbit IgG; Abiowell), anti-63 (1:1000; rabbit IgG; Abiowell), anti-TSG101 (1:1000; rabbit IgG; Abiowell), and anti-calnexin (1:1000; rabbit IgG; Abiowell).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-17A, IL-10, and IL-4 in each cell culture supernatants was measured using colorimetric sandwich ELISA kits (Proteintech, USA) according to the manufacturer's instructions. After coating microtiter plate wells with purified antibody, samples containing standards and serum were incubated with solid-phase antibody, forming complexes with biotinylated anti-IgG and streptavidin-HRP. The colored product's intensity, proportional to JAML concentration, was measured at 450 nm to calculate JAML concentration using a standard curve.

2.7. Statistical analysis

Descriptive analysis was shown as means ± standard deviation (SD). The difference between multiple groups was determined by the one-way analysis of variance (ANOVA) test, followed by Tukey's post hoc test for multiple comparisons. Statistical analysis was performed using GraphPad Prism 9. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of exosomes secreted from MPC-5

Exosomes were isolated from cell culture supernatant of LPS-treated MPC-5 cells. The average particle size determined by NTA was 137.4 ± 50.1 nm (Fig. 1A). The TEM images confirmed the presence of membranous-bound vesicles were round with typical sized ranging from 50 to 200 nm (Fig. 1B). The expression of exosomes markers CD9, CD63, and TSG101 were analyzed by Western blotting. As shown in Fig. 1C, the expressions of CD9, CD63, and TSG101 in exosomes were significantly higher than those in the cell culture supernatant, but lower than the expression in MPC-5 cells. Meanwhile, exosomes expressed the absence of calnexin, which is a protein marker related to the endoplasmic reticulum (Fig. 1C). These results indicate that the purified microvesicles primarily were exosomes. Furthermore, the expression of CD80, MHC II, and TLR-4 were detected among untreated MPC-5 cells, LPS-treated MPC-5 cells, exosomes of untreated MPC-5, and exosomes of LPS-treated MPC-5. According to previous study, LPS-treated podocytes could express MHC II, CD80, and TLR-4; while the purified exosomes expressed less levels of these factors (Fig. 1D).

Table 1
Sequence of the primers for the RT-qPCR.

Name	Primer sequence	Product length
GAPDH	F: 5′- ACAGCAACAGGGTGGTGGAC-3′ R: 5′- TTTGAGGGTGCAGCGAACTT -3′	252bp
RORC (ROR-γt)	F: 5′- AACTGCCCCATTGACCGAAC-3′ R: 5′- CGGCCAAACTTGACAGCATCTCG-3′	101bp
IL-17a	F: 5′- AGACTACCTCAACCGTTCCAC-3′ R: 5′- CACCAGCATCTTCTCGACCC-3′	229bp
IL-10	F: 5′- GTTCCCCTACTGTCATCCCC-3′ R: 5′- AGGCAGACAAACAATACACCA-3′	149bp
FOXP3	F: 5′- CTCCAATCCCTGCCCTTGACC-3′ R: 5′- ACATCATCGCCCGGTTTCCA-3′	130bp

Bp, base pair.

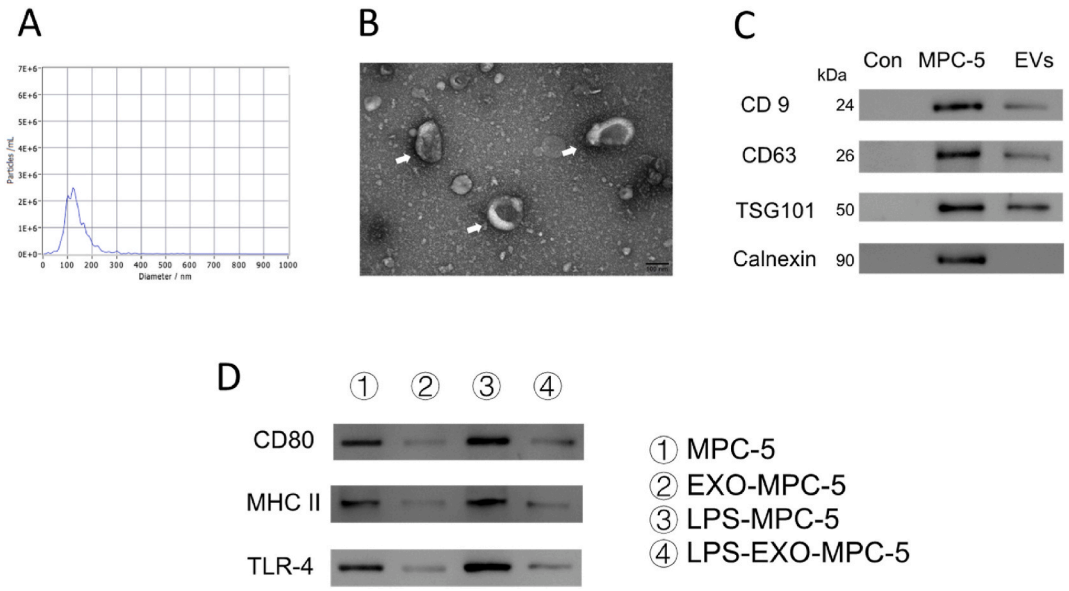

Fig. 1. Characterization of exosomes secreted from MPC-5. (A) Diagram of particle size distribution and concentration in exosomes analyzed by nanoparticles tracking analysis. (B) Representative transmission electron microscopy showing exosomes (arrowheads). Western blots analysis of exosomes markers (CD9, CD63, TSG101) (C) and CD80, MHC II, TLR-4 (D). The control samples were cell culture supernatant.

3.2. Exosomes secreted from LPS-treated MPC-5 could promote Th17 generation

Fig. 2 presents the schematic diagram of this study. Naïve $CD4^+$ T cells were co-cultured with LPS-treated BMDC (LPS-BMDC), LPS-treated MPC-5 (LPS-MPC-5), and exosomes derived from LPS-MPC-5 (LPS-EXO), respectively. As a control group, naïve $CD4^+$ T cells were cultured with exosomes from untreated MPC-5 (EXO). After 48 h, the percentage of Th17 cells ($CD4^+IL17^+$ cells) significantly increased in the LPS-EXO, LPS-BMDC, and LPS-MPC-5 gorups compared to the control groups (Fig. 3A–F). However, the elevation of Th17 cells in LPS-EXO group was lower than that in LPS-BMDC and LPS-MPC-5 groups (Fig. 3F).

Furthermore, the concentration of the Th17 cytokine IL-17A was compared among five groups using ELISA. The concentration of IL-17A in supernatants was significantly higher in the LPS-EXO, LPS-BMDC and LPS-MCP-5 groups compared to the EXO and control groups (Fig. 3G). RT-qPCR analysis of Th17-related transcription factor RORC and cytokine IL-17A confirmed the ELISA results, indicating that exosomes secreted from podocytes could promote the Th17 differentiation (Fig. 3H and I). The expression of IL-17A mRNA in the LPS-EXO group was significantly lower than in the LPS-MPC-5 groups, while there was no significant change in secretion level of IL-17A and expression of RORC mRNA between LPS-EXO and LPS-MPC-5 groups. (Fig. 3G–I).

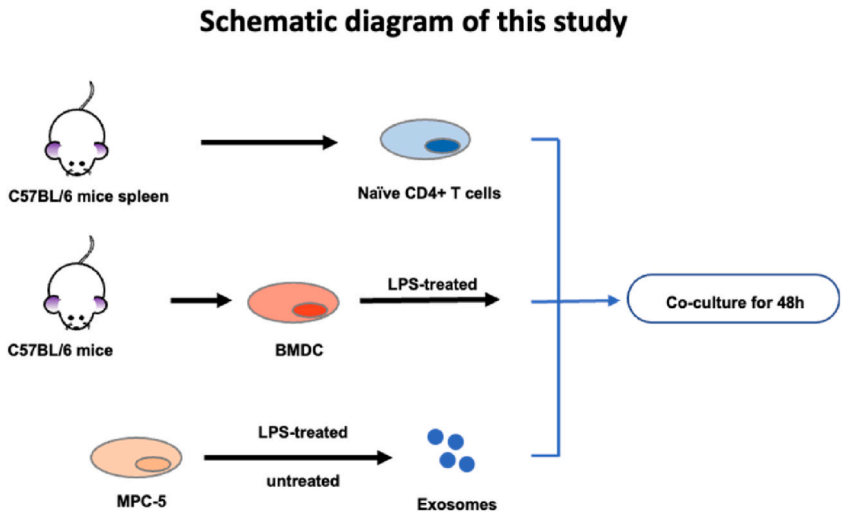

Fig. 2. Schematic diagram of this study.

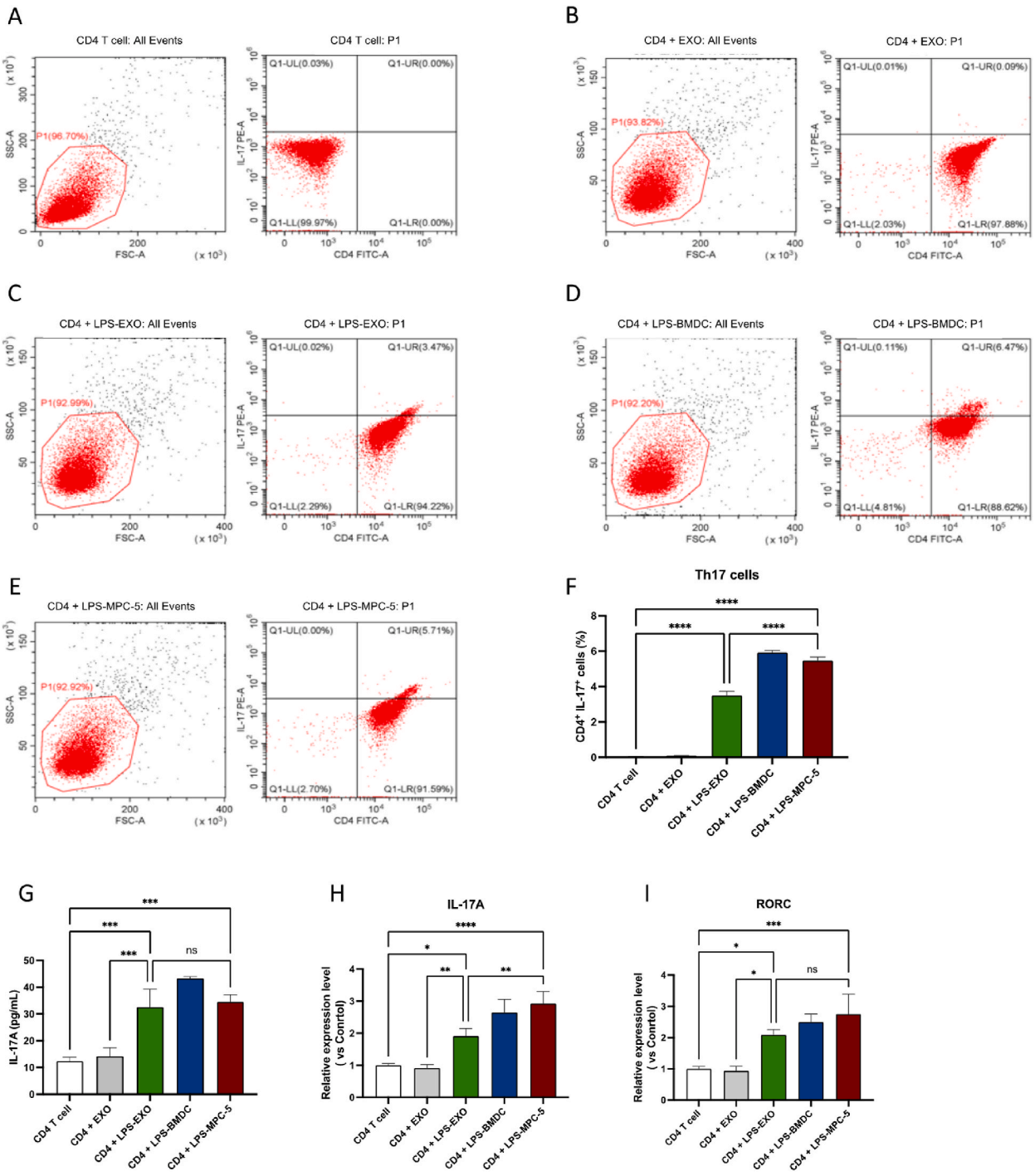

Fig. 3. The exosomes secreted from LPS-treated MPC-5 could promote naïve $CD4^+$ T cells transformed to Th17 cells. (A)–(E) Percentages of $CD4^+IL17^+$ cells after culturing for 48 h in gated $CD4^+$ T cells. F: The percentage of Th17 cells (means ± SD) in each group after simulation. (G) The concentration of IL-17A (means ± SD) in supernatants by ELISA. (H) and (I) The expression of IL-17A and RORC mRNA (means ± SD) by RT-qPCR. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$ (Tukey's test).

3.3. *Exosomes secreted from LPS-treated MPC-5 could promote Treg transformation*

After 48 h of co-cultured with naïve $CD4^+$ T cells, there was a significant increase in the percentage of Treg cells ($CD4^+CD25^+FOXP3^+$ cells) in the LPS-EXO, LPS-BMDC, and LPS-MPC-5 groups compared to the control groups (Fig. 3A–F). However,

the percentage of Treg cells in the LPS-EXO group was lower than in the LPS-MPC-5 group (Fig. 3F).

The concentration of Treg cell cytokines IL-4 and IL-10 was measured using ELSIA. The concentrations of IL-10 and IL-4 were higher in the LPS-BMDC and LPS-MPC-5 groups compared to the other three groups (Fig. 4G and H). The LPS-EXO group showed

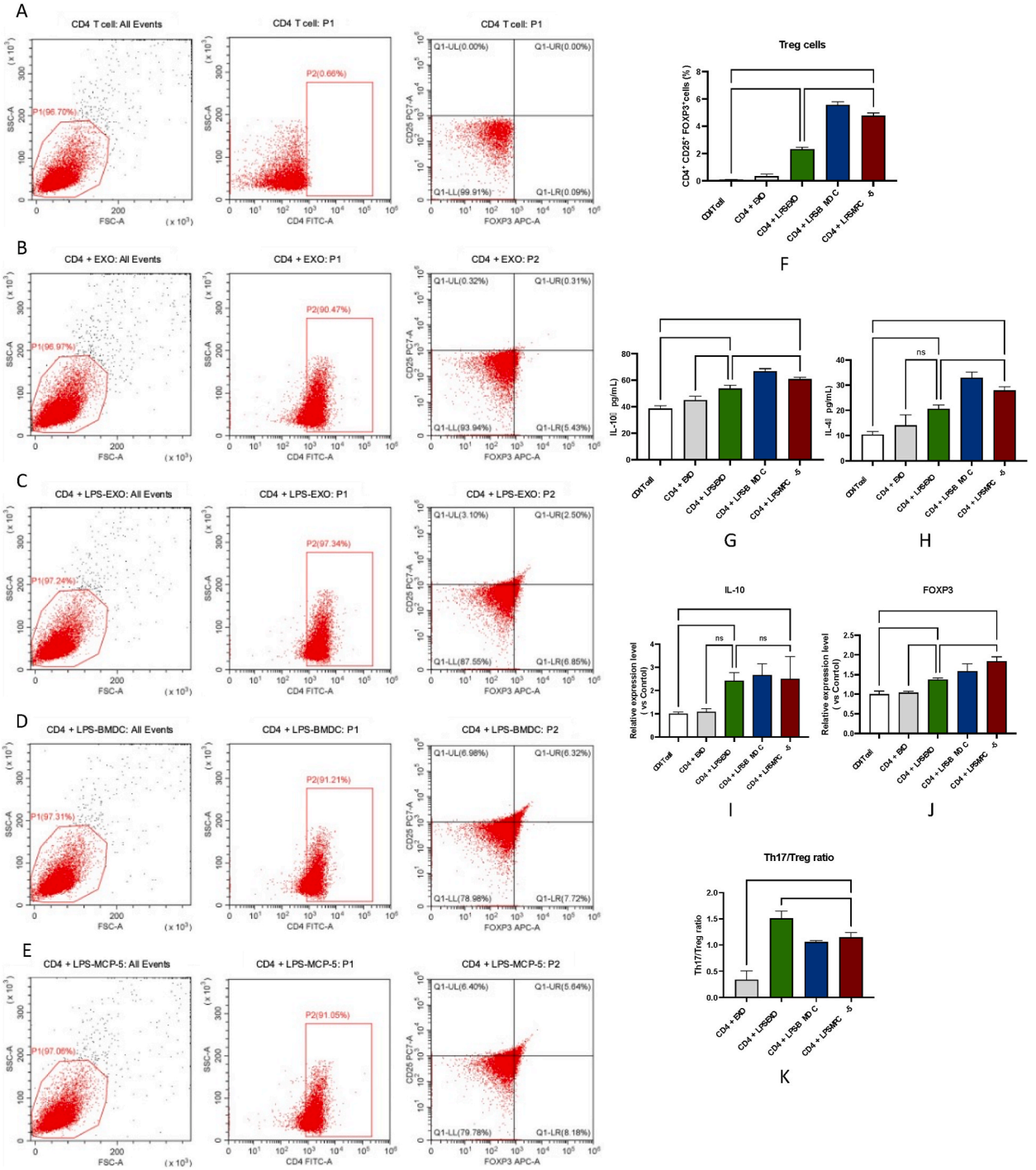

Fig. 4. The exosomes secreted from LPS-treated MPC-5 could promote naïve $CD4^+$ T cells transformed to Treg cells. (A)–(E) Percentages of $CD4^+CD25^+FOXP3^+$ cells after culturing for 48 h in gated $CD4^+$ T cells. (F) The percentage of Treg cells (means ± SD) in each group after simulation. (G) and (H) The concentration of IL-10 and IL-4 (means ± SD) in supernatants by ELISA. (I) and (J) The expression of IL-10 and FOXP3 mRNA (means ± SD) by RT-qPCR. (K) The Th17/Treg ratio (means ± SD) in each group after culturing for 48 h *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$ (Tukey's test).

higher levels of both IL-4 and IL-10 compared to the control group, while only exhibiting a higher level of IL-10 compared to the EXO group (Fig. 4G and H). In addition, the expression of Treg-related transcription factor FOXP3 and cytokine IL-10 was assessed by RT-qPCR. Consistent with the ELSIA results, the expressions of FOXP3 and IL-10 were increased in the LPS-BMDC and LPS-MPC-5 groups (Fig. 4I and J). The expression of FOPX3 in LPS-EXO group was higher than in the EXO group but lower than in the LPS-MPC-5 group (Fig. 4J). Although the LPS-EXO group showed increased expression of IL-10 compared to the control group, there were no significant changes of IL-10 expression between the LPS-EXO and EXO group, as well as between the LPS-EXO and LPS-MPC-5 groups (Fig. 4I).

3.4. Exosomes secreted from LPS-treated MPC-5 could regulate the Th17/Treg ratio

To investigate the potential role of exosomes in regulating the balance between Th17 and Treg cells, we analyzed the ratio of Th17/Treg in each experimental group. Interestingly, we found that the Th17/Treg ratio was significantly higher in the LPS-EXO, LPS-BMDC, and LPS-MPC-5 groups compared to the EXO group. Notably, despite the higher percentages of both the Th17 ($CD4^+IL17^+$) cells and Treg ($CD4^+CD25^+FOXP3^+$) cells in the LPS-MPC-5 group compared to the LPS-EXO group (Figs. 3F and 4F), the Th17/Treg ratio was relatively lower in the LPS-MPC-5 group than in the LPS-EXO group (Fig. 4K).

4. Discussion

Idiopathic nephrotic syndrome (INS) refers to a group of glomerular diseases that cause the typical triad of severe proteinuria, edema, and hypoalbuminaemia [21]. The incidence of INS varies widely, with a greater incidence affecting 1.15–16.9 persons per 100, 000 children globally [22]. Over the past two decades, our understanding of the pathogenesis of INS has been greatly influenced by the study of glomerular podocytes, the target cells involved in this condition. In this study, we successfully isolated exosomes secreted by podocytes, and demonstrated their ability to influence the Th17/Treg immune balance.

Since the 1970s, it has been recognized that the fundamental pathogenic mechanism of INS involves the dysregulation of T-cell immunity according to clinical data [23]. Subsequently, several alterations in T cell subpopulations have been documented. Th17 cells, a subset of $CD4^+$ T helper cells, play crucial roles in promoting inflammation and inducing autoimmune tissue damage by releasing pro-inflammation cytokines such as IL-17, IL-23, and tumor necrosis factor-α (TNF-α). Conversely, Treg cells have an anti-inflammation function and maintain tolerance to self-components by producing anti-inflammatory cytokines such as IL-10 and transforming growth factor-β1 (TGF-β1). An increase in Th17 cells along with a reduction in Treg cells has been consistently found in both pediatric and adult patients with the onset of INS [4,24,25]. A clinical study has demonstrated that a decrease in Treg cells and an increase in the Th17/Treg ratio may play the key role in the pathogenesis of idiopathic membranous nephropathy, with is the primary cause of INS in adults [26]. Additionally, lower baseline Treg levels and slow growth of Treg cells are associated with repeated relapses of INS [27,28]. Similarly, another study showed that decreased Treg levels at baseline could predict the incidence of early relapse following immunosuppressive therapy in a pediatric cohort of patients with INS [29].

It has been reported that BMDCs are more effective than B cells and macrophages in presenting antigens to naïve T cells [30,31]. LPS-stimulated BMDCs have the ability to induce naïve Th cells to differentiate into Th1, Th2, Th17, or Treg cells under the specific conditions [32,33]. Therefore, in this study, LPS-BMDCs were selected for co-culture with naïve $CD4^+$ T cells as a positive control group. Diseases that affect podocytes, which are highly specialized cells in the glomerulus that play a key role in the filtration system, often manifest with proteinuria or symptoms associated with proteinuria as the primary clinical manifestation [34,35]. In addition to genetic factors, podocyte injury can result from immunological, infectious (such as various virus infections), toxic (from numerous drugs and metals) and obesity related factors [36,37]. It has been demonstrated that naïve T cells can migrate to the kidney and differentiate into and activate Th17 cells in response to local stimuli [38,39]. Recent evidences suggest that podocytes may function as antigen-presenting cells (APCs) since they express immune system markers such as CD80 and MHC II [8,10,40,41]. Specifically, podocytes have the ability to activate local Th17 cells under inflammatory conditions [42]. In a previous study, we have reported that podocytes treated with LPS could alter the balance between Th17 and Treg cells *in vitro* [10]. Therefore, it is crucial to gain a better understanding of the molecular pathways within the podocytes and how this knowledge can provide insights into the potential mechanisms by which circulating substances can affect these pathways.

Exosome, a type of extracellular vesicle (EV), with an average size of approximately 100 nm, are actively secreted by almost all cells. Serving as signaling molecules, they play a vital role in mediating biological processes by facilitating cell-to-cell communication. They contain various constituents, including nucleic acids, proteins, lipids, amino acids, and metabolites [11]. The quantity and molecular composition of exosomes isolated from biological fluids can be utilized for disease identification. It has been demonstrated that EVs can present antigens through carrying pMHC-II complexes, as well as costimulatory and adhesion molecules, resulting in the specific activation of T cells, as observed in EVs extracted from B cells and DCs [19,43]. Exosomes produced by inflammatory infiltrating cells or resident cells are believed to contribute to immune system dysfunction in autoimmune and chronic inflammatory illnesses [44]. Exosomes derived from tumors could also modulate the immune response by inhibition cytotoxic $CD8^+$ T cells and natural killer cells, promoting Treg differentiation, and regulating macrophage polarization [45,46]. They achieve these effects through antigen-presenting functions similar to those of other cells. Previous studies suggest that exosomes could serve as a novel approach for delivering antigens to immune cells. For example, in a model of acute and chronic kidney injury model, exsomal miR-19b-3p derived from tubular epithelial has been shown to increase NF-kB activity by directly targeting SOCS-1, resulting in M1 macrophage activation [47]. Additional clinical research has demonstrated that exosomal miR-19b-3p in the urine from patients with diabetic nephropathy contributes to tubulointerstitial inflammation and may act as a potential biomarker for kidney disease [47]. Our previous [10] and recent studies have revealed that podocytes exhibit antigen-presenting functions not only by expressing surface

markers like CD80 or MHC II, but also through the release of exosomes that subsequently internalized by immune cells.

5. Conclusions

In summary, our study provides further insights into the role of exosomes delivered from LPS-treated podocytes in modulating the Th17/Treg cell balance in INS. Understanding the mechanisms underlying these immune alterations may lead to the development of novel therapeutic strategies for the treatment of INS. However, additional research is needed to explore the specific signal pathways and the molecules involved in these exosomes.

Ethics statement

The studies involving animals were reviewed and approved by the animal ethics committee of the Second Xiangya Hospital of Central South University.

Data availability

Data included in article/supp. Material/referenced in article.

Funding

This work was supported by the National Key R&D Program of China (No.2021YFC2702004), the Natural Science Foundation of Hunan Province (No.2021JJ40836), and the Scientific Research Launch Project for new employees of the Second Xiangya Hospital of Central South University.

CRediT authorship contribution statement

Yang Jia: Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Shiqiu Xiong:** Writing – original draft, Formal analysis, Data curation. **Haixia Chen:** Conceptualization. **Donghai Liu:** Conceptualization. **Xiaochuan Wu:** Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge all participants involving the collection of the baseline data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37866.

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