

Interleukin-6 via Toll-Like Receptor 3 Signaling Attenuates the Expression of Proinflammatory Chemokines in Human Podocytes

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Keywords

C-C motif chemokine ligand 5 · Interleukin-6 · Monocyte chemoattractant protein-1 · Podocytes · Toll-like receptor 3

Abstract

Background: Although toll-like receptor 3 (TLR3) signaling is involved in the development of certain chronic kidney diseases, the specific molecular mechanisms underlying inflammatory reactions via activation of TLR3 signaling in human podocytes remain unclear. Interleukin (IL)-6 is a pleiotropic cytokine associated with innate and adaptive immune responses; however, little is known about the implication of IL-6 via the activation of regional TLR3 signaling in the inflammatory reactions in human podocytes. **Methods:** We treated immortalized human podocytes with polyinosinic-polycytidylic acid (poly IC), an authentic viral double-stranded RNA, and assessed the expression of IL-6, monocyte chemoattractant protein-1 (MCP-1), and C-C motif chemokine ligand 5 (CCL5) using quantitative real-time reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay. To further elucidate the poly IC-induced signaling pathway, we subjected the cells to RNA interference against IFN- β and IL-6. **Results:** We found that the activation of TLR3 induced expression of IL-6, MCP-1, CCL5, and IFN- β in human podocytes. RNA interference experiments revealed that IFN- β was involved in the poly IC-induced expression of IL-6, MCP-1, and CCL5. Interestingly, IL-6 knockdown markedly increased the poly IC-induced expression of MCP-1 and CCL5. Further, treatment of cells with IL-6 attenuated the expression of CCL5 and MCP-1 mRNA and proteins. **Conclusion:** IL-6 induced by TLR3 signaling negatively regulates the expression of representative TLR3 signaling-dependent proinflammatory chemokines in human podocytes.

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Introduction

In clinical practice, viral infections are known to trigger the development of chronic kidney diseases (CKD) or worsen the pre-existing CKD [1, 2]. Recognition of the molecular pattern of viral pathogens by toll-like receptor 3 (TLR3) found in intracellular endosomes and subsequent immunoreactions is important in host antiviral defenses [3]. TLR3 signaling cascades activate the toll/interleukin (IL)-1 receptor-domain-containing adapter-inducing interferon- β -dependent pathways and the

subsequent release of inflammatory chemokines, cytokines, adhesion molecules, and eventually type I interferons [3]. Since the expression of TLR3 in resident glomerular cells is confirmed in biopsy specimens from patients with CKD [4, 5], regional antiviral host defenses via the activation of TLR3 signaling in the intrinsic glomerular cells have been postulated to be involved in the pathogenesis of some CKD types [4–6]. To date, considering the implications of TLR3 signaling in the pathogenesis of CKD, we treated cultured human mesangial cells (MCs) and glomerular endothelial cells (GECs) with polyinosinic-polycytidylic acid (poly IC), an authentic viral double-stranded RNA, and thereafter examined the expressions of representative proinflammatory chemokines and cytokines [6–12]. Notably, podocytes themselves also reportedly express TLR3 and its downstream signaling pathways, and these axes are presumed to be involved in the pathogenesis of certain CKD forms [13, 14].

Among the TLR3 signaling-induced functional molecules in resident glomerular cells, we recently reported that TLR3 signaling can induce IL-6 expression in GECs [11]. IL-6, a representative proinflammatory mediator, is known as a pleiotropic cytokine that regulates pro- and anti-inflammatory reactions in numerous cell types, including resident glomerular cells [15–17]. Considering the role of IL-6 in podocytes, it has been reported that high glucose exposure-induced IL-6 expression causes apoptosis in rat podocytes [18]. In contrast, IL-6-dependent cellular crosstalk between podocytes and GECs may modulate glomerular inflammation by reduced recruitment of neutrophils to the endothelium [19]. Thus, IL-6 may play a pivotal role in inflammation as well as prove to be protective or deleterious in the pathogenesis of CKD [17, 19]; however, the implication of IL-6 expression via TLR3 signaling in human podocytes remains unclear. Therefore, in the present study, we treated human podocytes with poly IC and evaluated the expression of IL-6 in this cell type.

Materials and Methods

Reagents

Insulin-transferrin-selenium-A supplement, penicillin-streptomycin, and siRNA against TLR3 (4427038) were purchased from Thermo Fisher Scientific (Asheville, MA, USA). RPMI-1640 and poly IC as a TLR3 ligand were obtained from Sigma (St. Louis, MO, USA). Small-interfering RNAs (siRNAs) against IL-6 (1299001) were purchased from Invitrogen (Frederick, MD, USA). A nonsilencing negative control siRNA (1027281) was purchased from Qiagen (Hilden, Germany). Lipofectamine RNAiMAX was supplied by Invitrogen (Carlsbad, CA, USA). An illustra RNA spin kit

Table 1. Oligonucleotide primers used for quantitative real-time PCR

cDNA	Primers
IL-6	F: 5'-ATGAACTCCTTCTCCACAAGC-3' R: 5'-AAGAGCCCTCAGGCTGGACTG-3'
CCL5	F: 5'-CTACTCGGGAGGCTAAGGCAGGAA-3' R: 5'-GAGGGGTTGATACGGCGGAAGC-3'
MCP-1	F: 5'-AAACTGAAGCTCGCACTCGC-3' R: 5'-ATTCTTGGGTTGTTGAGTGAGT-3'
IFN- β	F: 5'-CCTGTGGCAATTGAATGGGAGGC-3' R: 5'-CCAGGCACAGTGACTGTACTCCTT-3'
TLR3	F: 5'-CTCAGAAGATTACCAGCCGCC-3' R: 5'-CCATTATGAGACAGATCTAATG-3'
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3' R: 5'-ATGGTGGTGAAGACGCCAGT-3'

IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN- β , interferon- β ; TLR3, toll-like receptor 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

was obtained from GE Healthcare (Buckinghamshire, UK). Oligo (dT)₁₈ primer and additional oligonucleotide primers for polymerase chain reaction (PCR) were custom synthesized by Greiner (Atsugi, Japan). Sso-Advanced Universal SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). Moloney murine leukemia virus (MMLV) reverse transcriptase was obtained from Invitrogen. Recombinant human (r[h]) IL-6 and enzyme-linked immunosorbent assay (ELISA) kits for IL-6, C-C motif chemokine ligand 5 (CCL5), and monocyte chemoattractant protein-1 (MCP-1) were supplied by R&D Systems (Minneapolis, MN, USA). ELISA kits for IFN- α and IFN- β were purchased from PBL Assay Science (Piscataway, NJ, USA). siRNA against IFN- β is previously described [7–12]. Primer sequences are listed in Table 1.

Cell Culture

Immortalized human podocytes were kindly provided by Prof. Moin Saleem, the University of Bristol, UK. Cells were cultured in RPMI-1640 containing 10% FBS, 1% insulin-transferrin-selenium-A supplement, and penicillin-streptomycin (100 U/mL and 100 μ g/mL, respectively). Cells were incubated at 33°C for proliferation under a humidified atmosphere of 95% air and 5% CO₂ and then thermoswitched from 33 to 37°C for differentiation under nonpermissive conditions for 14 days. Medium was changed 3 times a week. Differentiated cells were incubated in 1% FBS for 24 h and then treated with poly IC. Poly IC was dissolved in phosphate-buffered saline, pH 7.4, and the cells were treated with 10–50 μ g/mL poly IC for up to 24 h.

In RNA interference experiments, cells were transfected in the 6-well plates with 20 pmol of siRNA (against TLR3, IFN- β , IL-6, or nonsilencing negative control siRNA) per well, using the Lipofectamine RNAiMAX reagent according to the supplier's protocol. The cells were 80–90% confluent at the time of transfection. After 48-h incubation, the cells were treated with poly IC as indicated. r(h) IL-6 was added to cells at 40 ng/mL, and cells were stimulated with 30 μ g/mL poly IC.

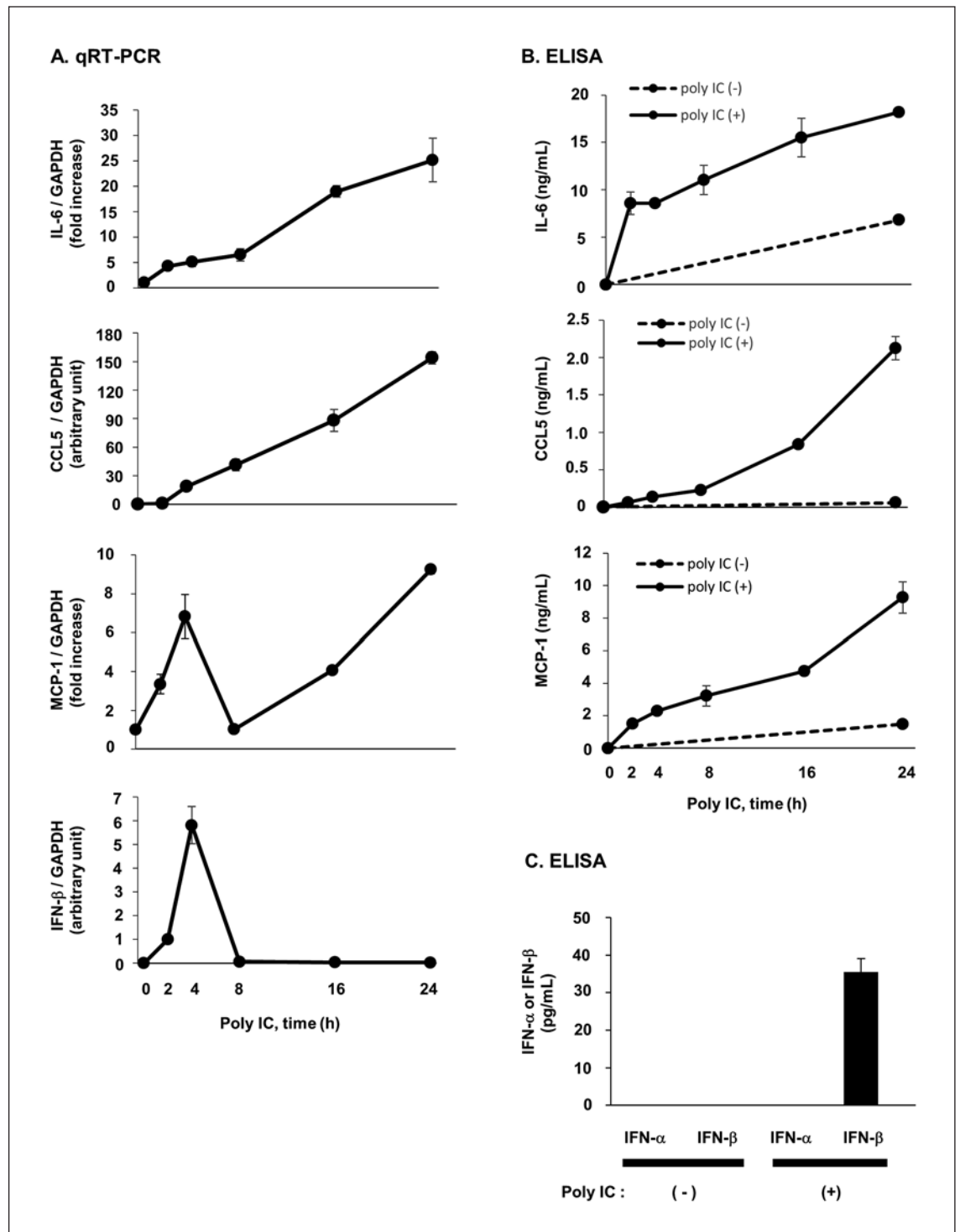


Fig. 1. Poly IC induces the expression of IL-6, CCL5, MCP-1, and IFN- β in cultured human podocytes in a time-dependent manner. Podocytes were cultured and treated with 30 μ g/mL poly IC up to 24 h. After 24-h incubation, RNA was extracted from the cells. **A** cDNA was synthesized, and the expression of IL-6, CCL5, MCP-1, and IFN- β mRNA was estimated using qRT-PCR analysis. After 24-h incubation, the conditioned medium was collected, and the

cells were lysed. **B** The concentration of IL-6, CCL5, and MCP-1 protein in the collected medium was measured using ELISA kits. **C** IFN- α protein was not detected by ELISA at the time point of after 24-h poly IC stimulation. The data are presented as means \pm standard deviation ($n = 3$). Poly IC, polyinosinic-polycytidylic acid; IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN- β , interferon- β .

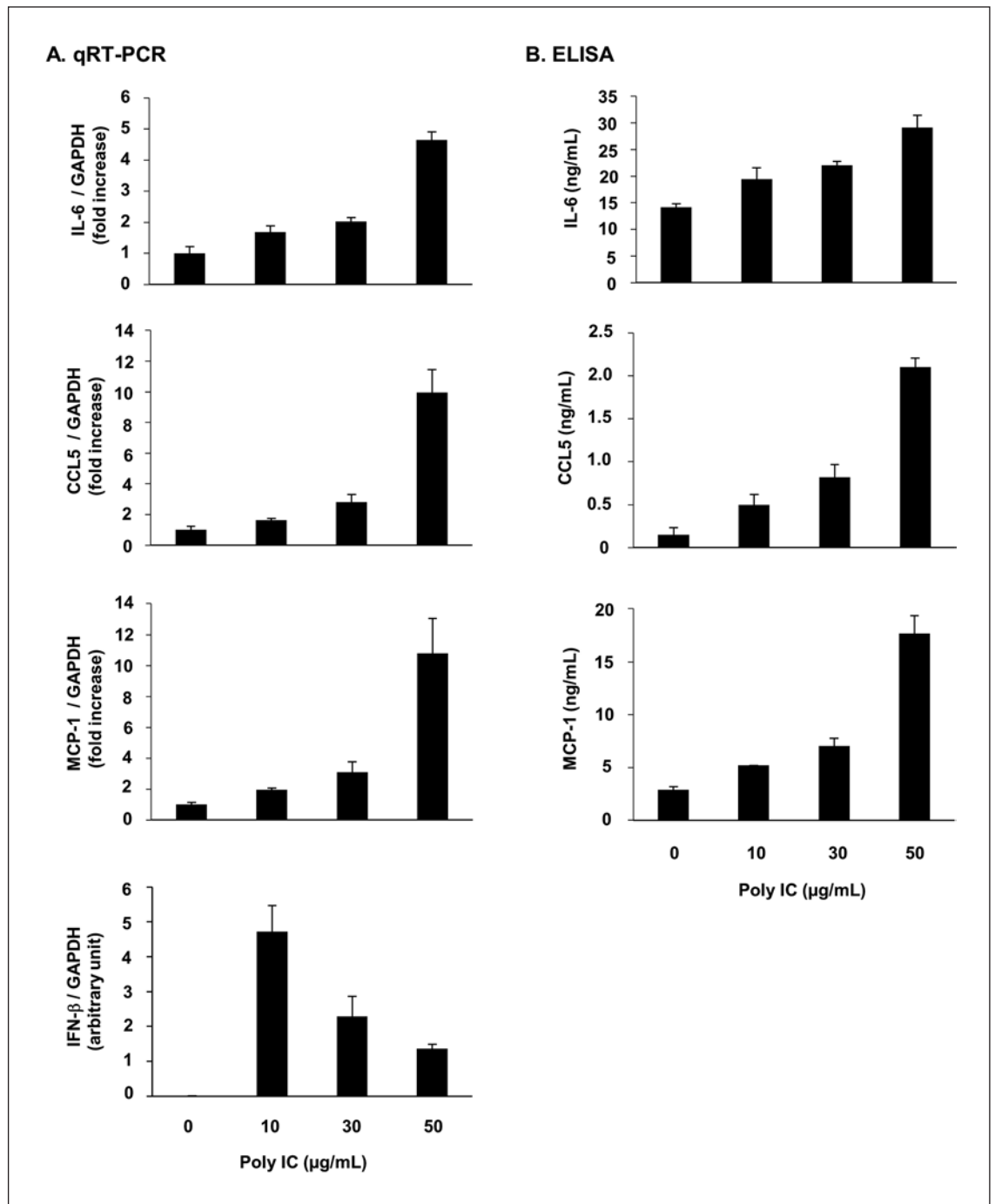
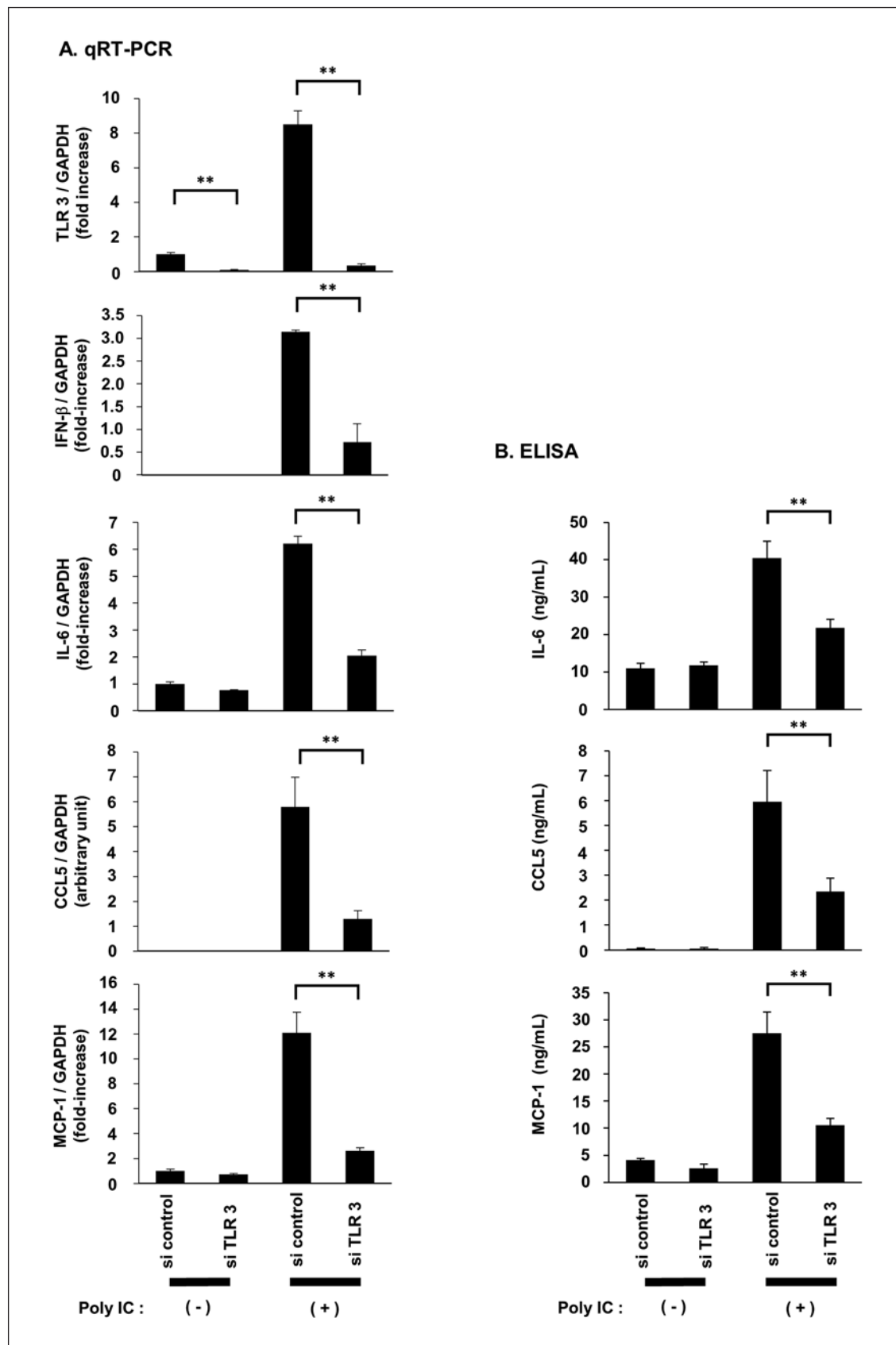


Fig. 2. Poly IC induces the expression of IL-6, CCL5, MCP-1, and IFN- β in cultured human podocytes in a concentration-dependent manner. Podocytes were cultured and treated with 10–50 μ g/mL poly IC for up to 24 h. **A** The expressions of IL-6, CCL5, MCP-1, and IFN- β mRNA were examined by qRT-PCR. **B** The concentra-

tion of IL-6, CCL5, MCP-1, and IFN- β proteins was estimated by ELISA. The data are presented as means \pm SD ($n = 3$). Poly IC, polyinosinic-polycytidylic acid; IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN- β , interferon- β .



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Quantitative Real-Time Reverse Transcription PCR Analyses

Total cellular RNA was extracted from the cells using an illustra RNAspin kit. Single-stranded cDNA was synthesized from 1 µg of total RNA using oligo (dT)₁₈ primer and MMLV reverse transcriptase. The cDNA for IL-6, CCL5, MCP-1, IFN-β, TLR3, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using Sso-Advanced Universal SYBR Green Supermix. Data were analyzed and expressed in comparison with GAPDH. The sequences of the primers used are shown.

ELISA

The concentrations of IL-6, CCL5, MCP-1, IFN-α, or IFN-β protein in the cell-conditioned medium were measured using the ELISA kits according to the manufacturer's protocol.

Statistical Analysis

All experiments were performed at least 3 times. That was, 3 independent experiments, and each experiment was done with 3 samples per group. Values are reported as means ± standard deviation. Differences between groups were analyzed using Student's *t* test. A *p* value <0.05 was considered as statistically significant. All analyses were carried out using GraphPad Prism software version 7 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Poly IC Induces the Expression of IL-6, CCL, MCP-1, and IFN-β in Cultured Human Podocytes

Treatment of cultured human podocytes with poly IC induced the expression of IL-6, CCL5, MCP-1, and IFN-β in a concentration-dependent manner (Fig. 1). The expression of GAPDH was not influenced by poly IC. IL-6 and CCL5 mRNA expression gradually increased up to 24 h after poly IC stimulation. MCP-1 and IFN-β mRNA expression rapidly increased after poly IC stimulation, peaked at 4 h, and decreased and returning to the basal level at 8 h, after which only MCP-1 mRNA increased again up to 24 h (Fig. 1A). IL-6, CCL5, and MCP-1 pro-

tein accumulated in the conditioned medium for up to 24 h in a time-dependent manner (Fig. 1B). IFN-α protein was not detected by ELISA at the time point of after 24-h poly IC stimulation (Fig. 1C).

Poly IC also induced the expression of IL-6, CCL5, MCP-1, and IFN-β in a concentration-dependent manner (Fig. 2). After poly IC stimulation, podocytes released higher levels of IL-6, CCL5, MCP-1, and IFN-β compared to the unstimulated cultures. When the cells were treated with various concentration of poly IC (10–50 µg/mL), the expression of IL-6, CCL5, and MCP-1 mRNA (Fig. 1A) and protein (Fig. 1B) was induced in a concentration-dependent manner. The expression of IFN-β mRNA peaked with 10 µg/mL poly IC stimulation (Fig. 2A).

TLR3 Is Involved in IFN-β, IL-6, CCL5, and MCP-1 Induction by Poly IC

RNA interference against TLR3 significantly inhibited the expression of mRNAs for IFN-β, IL-6, CCL5, and MCP-1 (Fig. 3A). We confirmed that protein expression of IL-6, CCL5, and MCP-1 was decreased by RNA interference against TLR3 (Fig. 3B). Effective knockdown of TLR3 mRNA by RNA interference was confirmed by qRT-PCR (Fig. 3A).

IFN-β Involvement in the Poly IC-Induced Expression of IL-6, CCL5, and MCP-1

To evaluate the role of IFN-β in poly IC-induced IL-6, CCL5, and MCP-1 expressions, we next performed the RNA interference experiments to knockdown the expression of IFN-β. Cell viability was evaluated using the trypan blue exclusion method. The viability of cells was >90% even after the transfection of cells with siRNAs. Effective knockdown of IFN-β expression was confirmed by ELISA (Fig. 4A). Knockdown of IFN-β significantly in-

Fig. 3. Knockdown of TLR3 diminishes the poly IC-induced expression of IFN-β, IL-6, CCL5, and MCP-1 in cultured human podocytes. The cells were transfected with siRNA against TLR3 or a nonsilencing negative control siRNA and were incubated for 48 h. Then, the cells were stimulated with 30 µg/mL poly IC. RNA was extracted from the cells after 4 h of incubation for the analysis of IFN-β or 24 h of incubation for the analysis of TLR3, IL-6, CCL5,

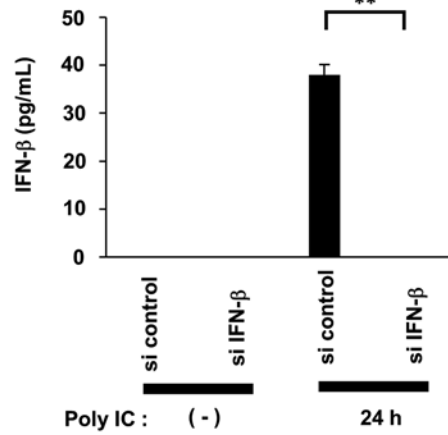
and MCP-1. **A** The expressions of TLR3, IFN-β, IL-6, CCL5, and MCP-1 mRNAs were examined by qRT-PCR analysis. **B** The concentration of IL-6, CCL5, and MCP-1 proteins was estimated by ELISA analysis. The data are means ± SD (*n* = 3, ***p* < 0.01). TLR3, toll-like receptor 3; poly IC, polyinosinic-polycytidylic acid; IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN-β, interferon-β.

Fig. 4. IFN-β is involved in poly IC-induced expression of IL-6, CCL5, and MCP-1 in cultured human podocytes. Cells were transfected with siRNA against IFN-β or a nonsilencing negative control siRNA. After 48-h incubation, the cells were stimulated with 10 µg/mL poly IC for up to 24 h. **A** To confirm the efficacy of siRNA against IFN-β, the expression of IFN-β protein was measured by

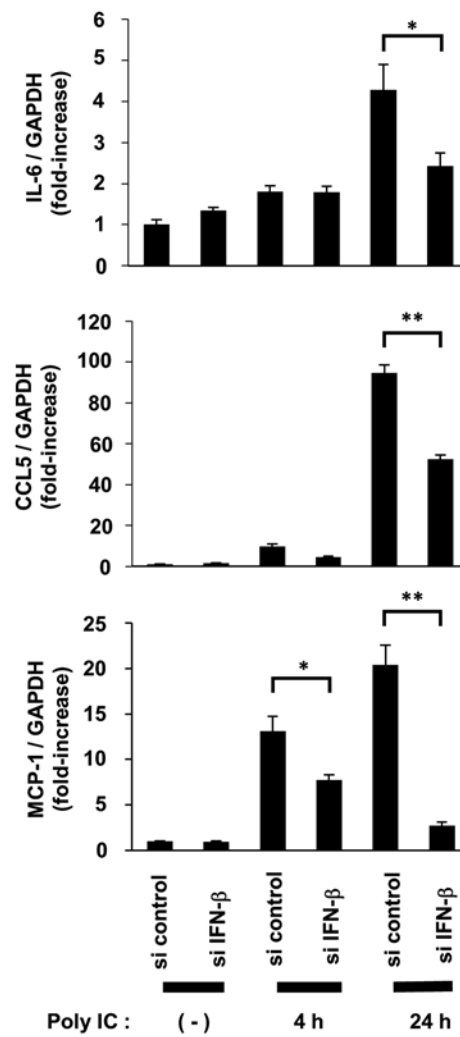
ELISA. The mRNA (**B**) and protein (**C**) expressions of IL-6, CCL5, and MCP-1 were examined as in Figure 2. The data are presented as means ± SD (*n* = 3, **p* < 0.05, ***p* < 0.01). IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN-β, interferon-β.

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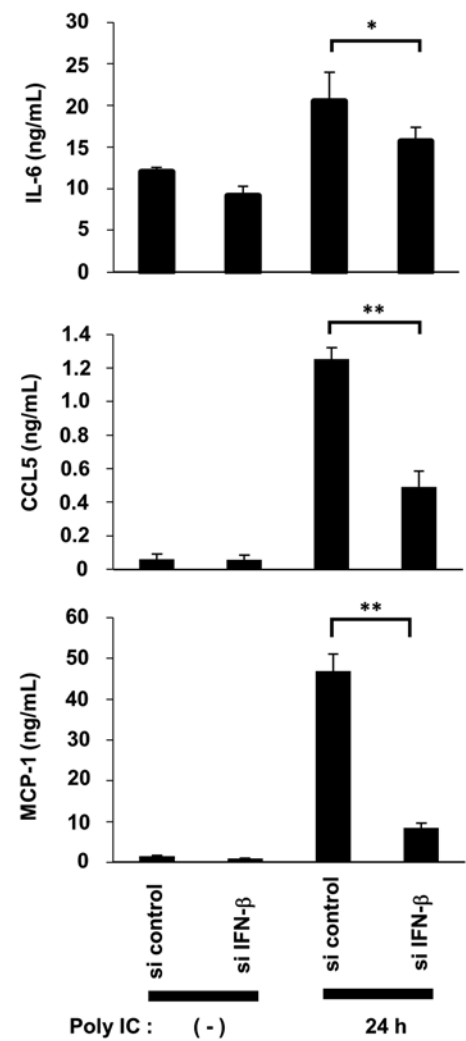
A. ELISA



B. qRT-PCR

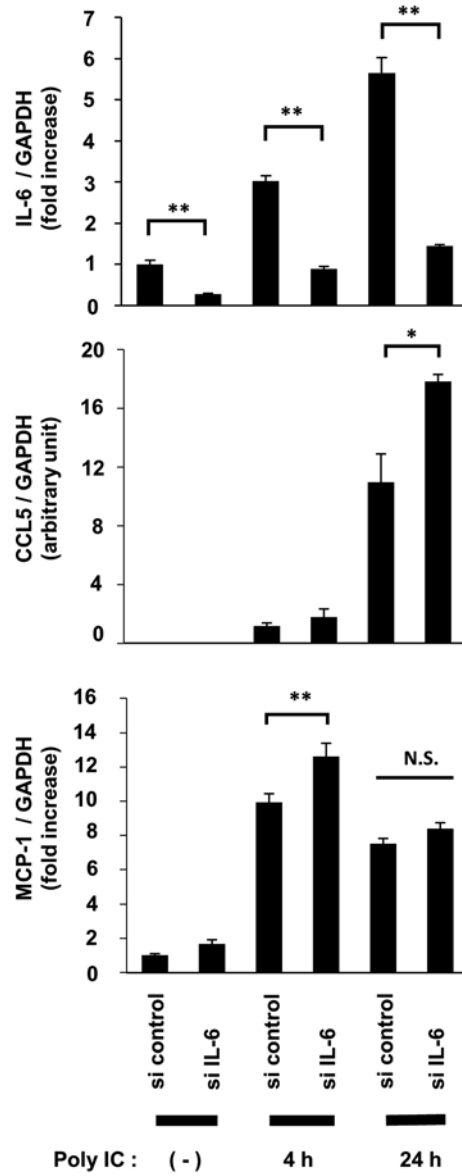


C. ELISA

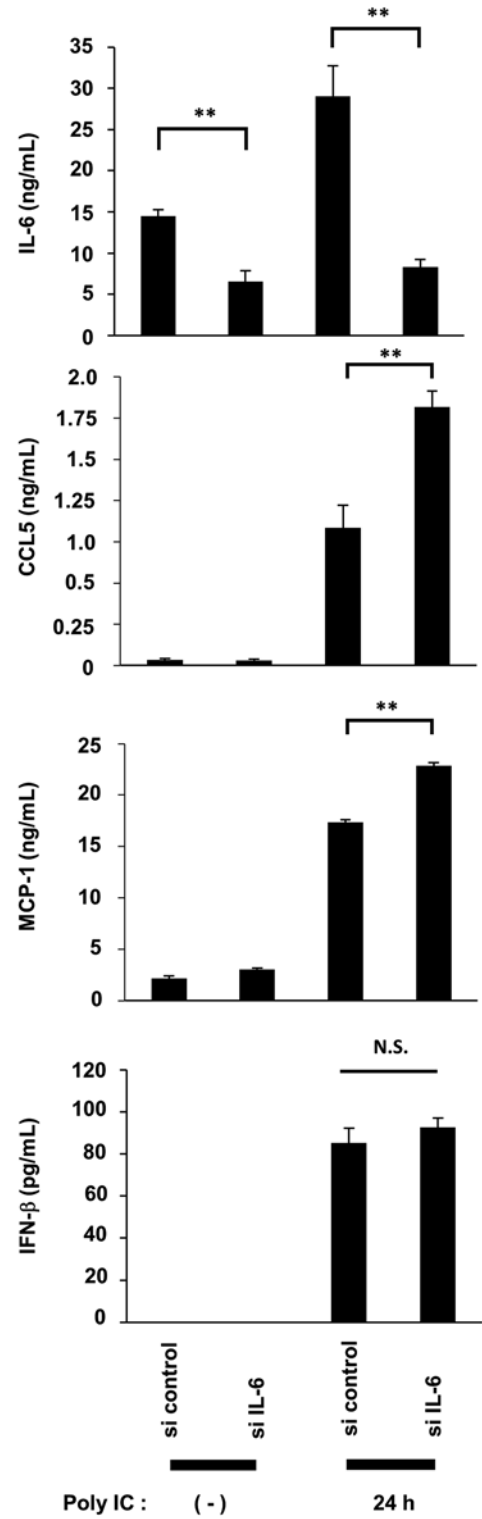


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A. qRT-PCR



B. ELISA



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hibited IL-6, CCL5, and MCP-1 mRNA (Fig. 4B) and protein (Fig. 4C) expression after 24-h poly IC stimulation. The expression of MCP-1 mRNA was decreased by knockdown of IFN- β significantly also after 4-h poly IC stimulation.

Knockdown of IL-6 Increases the Poly IC-Induced Expression of MCP-1 and CCL5 in Cultured Human Podocytes

To examine the effect of poly IC-induced IL-6 chemokine expression, we carried out siRNA targeting IL-6. Effective knockdown of IL-6 by RNA interference was confirmed by qRT-PCR and ELISA. Knockdown of IL-6 resulted in the significant increase of the expression of CCL5 mRNA (Fig. 5A) and protein (Fig. 5B). Knockdown of IL-6 resulted in increased expression of MCP-1 mRNA after 4-h poly IC treatment (Fig. 5A) and MCP-1 protein after 24 h poly IC treatment (Fig. 5B). However, knockdown of IL-6 had no effect on poly IC-induced increases in IFN- β protein level.

r(h) IL-6 Decreases the Poly IC-Induced Expression of MCP-1 and CCL5 in Cultured Human Podocytes

To further examine the effect of IL-6 on the expression of poly IC-induced chemokines, we next performed the treatment of cells with r(h) IL-6. Treatment of cells with r(h) IL-6 attenuated the expression of CCL5 and MCP-1 mRNA (Fig. 6A) and protein (Fig. 6B) after 24-h poly IC stimulation. The expression of MCP-1 mRNA was decreased significantly by 4-h poly IC stimulation. r(h) IL-6 had no effect on poly IC-induced increase in IFN- β protein level.

Discussion

IL-6 is a pleiotropic cytokine that regulates pro- and anti-inflammatory reactions in numerous cell types [15–17, 20]. In resident glomerular cells, IL-6 can be produced by MCs, GECs, and podocytes and is involved in the pathogenesis of CKD [11, 17, 20]. Recently, we reported

that the expression of IL-6 is induced by activating TLR3 and its signaling pathway, which is tightly regulated in human GECs [11]; however, the specific mechanisms underlying the induction of IL-6 production via TLR3 signaling in human podocytes remain unknown. Thus, in the present study, we aimed to examine these mechanisms and found that poly IC treatment induced the expression of IL-6 in human podocytes in a time- and concentration-dependent manner. Experiments using siRNA confirmed that this response was mediated by signaling via the TLR3/IFN- β axis, which is consistent with the results of our previous studies using human MCs and GECs [4–11]. Concerning type I IFNs, our previous studies using cultured human MCs and GECs demonstrated that IFN- β , but not IFN- α , synthesized de novo following TLR3 activation, is crucial in succeeding regional inflammatory cascades [3–12]. On the other hand, we found poly IC-stimulated expression of IFN- α in podocytes remained negative in our experimental setting. Recently, Migliorini et al. [21] reported that IFN- β specifically promotes podocyte loss by inducing mitotic catastrophe of podocytes and both IFN- α and IFN- β impair the differentiation of renal progenitors into mature podocytes. In the present study, protein levels of MCP-1 and CCL5 induced by poly IC were lesser owing to the increase in IL-6 (see Fig. 2B). Furthermore, knockdown of IL-6 significantly increased poly IC-induced MCP-1 and CCL5 in both messenger RNA and protein levels, thereby suggesting that TLR3 signaling-induced IL-6 expression negatively regulates the expression of MCP-1 and CCL5 in human podocytes, although this theory remains speculative.

Considering the implication of toll/interleukin (IL)-1 receptor-domain-containing adapter-inducing interferon- β -dependent pathway-related TLR signaling in podocytes, the blockade of TLR4 signaling can prevent apoptosis in diabetic nephropathy mice models [22]. Shimada et al. [13] previously reported that activation of TLR3 signaling increased the expression of cathepsin L, decreased synaptopodin expression, and resulted in actin reorganization, leading to podocyte injury. In con-

Fig. 5. Knockdown of IL-6 increases the poly IC-induced expression of CCL5 and MCP-1 in cultured human podocytes. The cells were transfected with siRNA against IL-6 or a nonsilencing negative control siRNA and incubated for 48 h, before being stimulated with 10 μ g/mL poly IC. **A** After an additional 4 or 24 h of incubation, RNA was extracted from the cells and was analyzed by qRT-PCR. After an additional 24-h incubation, the conditioned medi-

um was collected. **B** The concentration of IL-6, CCL5, MCP-1, and IFN- β protein was measured by ELISA. **C** The expression of IL-6 mRNA was measured by qRT-PCR to confirm the efficacy of siRNA against IL-6. The data are presented as means \pm SD ($n = 3$, ns, nonsignificant, $*p < 0.05$, $**p < 0.01$). Poly IC, polyinosinic-polycytidylic acid; IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN- β , interferon- β .

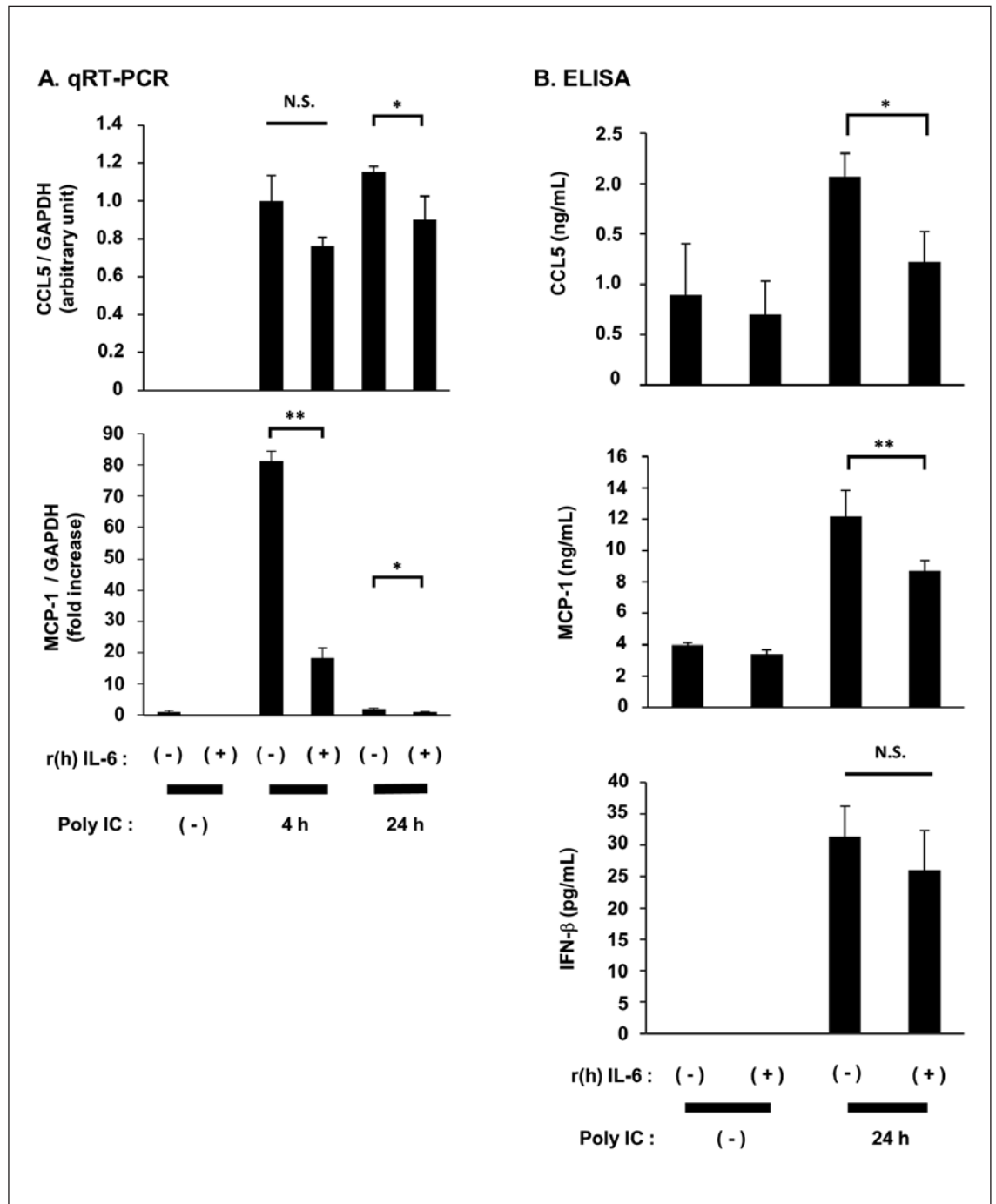


Fig. 6. Treatment of cells with r(h) IL-6 decreases the poly IC-induced expression of CCL5 and MCP-1 in cultured human podocytes. r(h) IL-6 was added to cells at 40 ng/mL, and cells were stimulated with 30 μ g/mL poly IC. **A** After an additional 4 h or 24 h of incubation, RNA was extracted from the cells, and qRT-PCR analysis was performed. **B** After an additional 24 h of incubation, the conditioned medium was collected, and the con-

centration of CCL5, MCP-1, or IFN- β protein was measured by ELISA. The data are means \pm SD ($n = 3$, ns, nonsignificant, $*p < 0.05$, $**p < 0.01$). Poly IC, polyinosinic-polycytidylic acid; IL-6, interleukin-6; CCL5, chemokine ligand 5; MCP-1, monocyte chemoattractant protein-1; IFN- β , interferon- β ; r(h), recombinant human.

trast, notably, TLR3 signaling has both protective and deleterious effects in the pathogenesis of viral infections [23]. Considering IL-6 in podocytes, it has been reported that high glucose exposure-induced IL-6 expression is involved in the development of apoptosis of rat podocytes [18], whereas IL-6-dependent cellular crosstalk between podocytes and GECs may modulate glomerular inflammation via neutrophil recruitment [19]. Thus, once IL-6 appears via TLR3 signaling activation associated with viral infections in podocytes, this functional molecule may play a pivotal role in the glomerular inflammation.

Lee et al. [24] reported that in podocytes, transforming growth factor- β -induced MCP-1 expression enhances cellular mobility, rearranges actin cytoskeleton, and increases podocyte permeability to albumin, and these effects are blocked by a neutralizing anti-MCP-1 antibody. Moreover, it has been reported that MCP-1 and CCL5 cause podocyte injury by activating the tumor necrosis factor- α /nuclear factor- κ B pathway, which is postulated in the viral infection-induced system, and this system may be exaggerated by virus-induced nuclear factor- κ B dysregulation [25]. Thus, attempts to improve the imbalance in expression levels of functional molecules, including MCP-1 and CCL5, may partly prevent podocyte injury in CKD. Therefore, further detailed studies on the implication of TLR3-induced IL-6 in podocytes are required to elucidate specific mechanism of CKD development or the exaggeration of pre-existing CKD following viral infections.

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Statement of Ethics

This article does not contain any studies with human participants or animals performed by any of the authors which otherwise require ethical approval.

Conflict of Interest Statement

All the authors have declared no competing interest.

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Author Contributions

S.W., T.I., and H.T. designed the study; H.U., T.I., T.A., S.W., and K.T. performed experiments; H.U. and H.T. wrote the manuscript; S.K., K.S., and T.M. gave technical support and conceptual advice. All authors read and approved the final manuscript.

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