Major histocompatibility complexes are up-regulated in glomerular endothelial cells via activation of c-Jun N-terminal kinase in 5/6 nephrectomy mice

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Background and Purpose: This study aims to explore the mechanism underlying the up-regulation of major histocompatibility complex (MHC) proteins in glomerular endothelial cells in 5/6 nephrectomy mice.

Experimental Approach: C57/BL6 mice were randomly allocated to sham-operated (2K) and 5/6 nephrectomy (5/6Nx) groups. Mouse splenic lymphocytes, from either syngeneic or allogeneic background, were injected into 5/6Nx mice after total body irradiation. Human glomerular endothelial cells (HGECs) were cultured for experiments in vitro. Western blots, PCR, immunohistochemical and fluorescent staining were used, along with assays of tissue cytokines, lymphocyte migration and renal function.

Key Results: Four weeks after nephrectomy, expression of both mRNA and protein of MHC II, CD80, and CD86 were increased in 5/6Nx glomerular endothelial cells. After total body irradiation, 5/6Nx mice injected with lymphocytes from Balb/c mice, but not those from C57/BL6 mice, exhibited increased creatinine levels, indicating that allograft lymphocyte transfer impaired renal function. In HGECs, the protein levels of MHC and MHC Class II transactivator (CIITA) were increased by stimulation with TNF-α or IFN-γ, which promoted human lymphocytes movement. These increases were reduced by JNK inhibitors. In the 5/6Nx mice, JNK inhibition down-regulated MHC II protein in glomerular endothelial cells, suggesting that JNK signalling participates in the regulation of MHC II protein.

Conclusion and Implications: Chronic inflammation in mice subjected to nephrectomy induces the up-regulation of MHC molecules in glomerular endothelial cells. This up-regulation is reduced by inhibition of JNK signalling.
1 | INTRODUCTION

Major histocompatibility complex (MHC) proteins are constitutively expressed on the cell surface and are essential for the acquired immune system to recognize foreign molecules. Professional antigen-presenting cells, including macrophages, B lymphocytes, and dendritic cells, display foreign antigens with MHCs on their surfaces. Under pathological conditions, increased levels of MHC complexes, mainly MHC Class II, are also observed in endothelial cells (Taflin et al., 2011), including those in cardiac (Rose, Coles, Griffin, Pomerance, & Yacoub, 1986), renal (Fuggle, McWhinnie, Chapman, Taylor, & Morris, 1986), and hepatic (Steinhoff, Wonigeit, & Pichlmayr, 1988) allografts. In long-term murine cardiac allografts, enhanced levels of endothelial MHC II molecules accelerates the development of graft arteriosclerosis (Hasegawa, Becker, Nagano, Libby, & Mitchell, 1998). Inhibiting endothelial MHC II prevents the migration of lymphocytes and mitigates acute rejection (Abrahimi et al., 2016). Thus, endothelial cells play a crucial role in the initiation of the immune response to the allograft. The mechanisms underlying the presentation of antigen by endothelial cells are still under debate, although endothelial cells are the first target in graft rejection (Vanhoutte, Zhao, Xu, & Leung, 2016).

Apart from immune surveillance from the recipients, changes in haemodynamic forces and local inflammatory responses are the most important mechanisms in allograft organs. Acute ischaemia-reperfusion injury is unavoidable in the process of transplantation, in which enhanced oxidative stress and increased inflammatory responses are major factors, resulting in delayed allograft function. Chronic haemodynamic changes, mainly hyper-perfusion in the allograft liver or kidney, are less studied. It is reported that (Jin et al., 2017) 4 weeks after nephrectomy, protein levels of endothelial NOS (eNOS) were increased in the remnant kidney, and was associated with reduced microvascular resistance, thus indicating that remnant glomeruli were subjected to hyper-perfusion. However, 4 months later, these mice suffer from renal failure, indicating that the eNOS up-regulation did not compensate for renal failure. It also suggests that the effects of local inflammatory responses outweigh those of chronic hyper-perfusion in the progress of renal failure. Elevated levels of proinflammatory cytokines are observed in the early stage of renal mass removal, as well as in renal ischaemia–reperfusion injury (Zhang, Tang, Li, & Wang, 2018) and cisplatin nephrotoxicity models (Ramesh & Reeves, 2002). The role of IFN-γ in the regulation of MHC complexes has been well studied, but has been mainly focused on macrophages and dendritic cells (Konieczny et al., 1998; Taflin et al., 2011; Wedgwood, Hatam, & Bonaga, 1988). Thus, the present study aimed to test the hypothesis that chronic inflammation causes glomerular endothelial dysfunction, allowing endothelial cells to play a role in the initiation of immune responses by antigen-presenting cells.

What is already known

- Professional antigen-presenting cells, including macrophages, B lymphocytes, and dendritic cells, display foreign antigens with MHCs.

What this study adds

- Under pathological conditions, endothelial cells also present the MHC complex on the cell surface.
- Inhibitors of JNK down-regulate MHC molecules induced by chronic inflammation in glomerular endothelial cells.

What is the clinical significance

- Microvascular endothelial cells are the first target of the immune responses.
- Regulation by MAPK–JNK of MHC expression sheds light on treatments in renal disease.

We have also examined the mechanisms underlying this action of endothelial.

2 | METHODS

2.1 | Animal experiments

All animal care and experimental protocols were approved by the Zhongshan Hospital Animal Care Committee (#ZS2015-005). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). C57/BL6 and BALB/c6 male mice weighing 22–25 g were purchased from Jiesijie Experimental Animals (Shanghai, PRC) and housed in specific pathogen-free cages with vent rack system in the Laboratory Animal Unit of Zhongshan Hospital (Shanghai, PRC). Four to five mice were housed in cages (29*19*13) containing bedding made of dried wood chips under clean and temperature-controlled conditions. The mice were fed regular chow and given free access to water.

Ten-week-old C57/BL6 mice were used in the present study. These mice were randomly allocated to sham-operated (2K, n = 38) and 5/6 nephrectomy (5/6Nx, n = 72) groups. The mice were
anaesthetized with pentobarbitone (50 mg·kg\(^{-1}\), i.p.). To achieve 5/6 nephrectomy, subtotal renal ablation of the left kidney was conducted by amputation of both renal poles. One week later, the mice were subjected to a right nephrectomy via a dorsal lumpectomy incision. After the surgery, mice were given butorphanol (i.p., 2 mg·kg\(^{-1}\)) to relieve pain. After 4 weeks, the mice were killed with an overdose of pentobarbitone (100 mg·kg\(^{-1}\), i.p.). Blood samples were prepared in tubes with 1.5 mg·ml\(^{-1}\) of EDTA. Mouse renal cortex tissue was collected for further examination.

JNK inhibitors were administered 1 week after surgery. SP600125 (10 mg·kg\(^{-1}\), dissolved in DMSO, \(n = 9\)) was administered i.p. five times per week during 3 weeks (Rahman, Zhang, Mody, Su, & Das, 2012; Takahashi et al., 2013). C-Jun peptide (5 mg·kg\(^{-1}\), dissolved in PBS, \(n = 10\)), a peptide that specifically inhibits JNK, was injected into mice i.p. every 3 days during 3 weeks (Kim et al., 2016).

A total of 114 C57/BL6 mice were used in the present study, 110 for animal experiments and four for splenic lymphocytes isolation. Four BALB/c6 mice were used for splenic lymphocytes isolation. Animals were randomized for treatment. Data collection and evaluation of all experiments were performed blindly of the group identity.

### 2.2 | Total body irradiation of mice

Thirty-eight mice were subjected to total body irradiation (\(n = 15\) in 2K group and \(n = 23\) in 5/6Nx group). One week after surgery, the C57/BL6 mice were placed in a closed chamber for total body irradiation. They were exposed to \(^{137}\)Cs γ-ray at a rate of 1.02 G·min\(^{-1}\) for 5 min (Cui et al., 2002); this irradiation regimen was optimized in preliminary experiments (data not shown). After irradiation, the mice were treated with amoxicillin (130 mg·kg\(^{-1}\)·day\(^{-1}\), RuìYang, Shandong, PRC) in drinking water, for 2 weeks to minimize bacterial infection. There were no apparent bodyweight losses in the mice after irradiation (Figure S1).

### 2.3 | Lymphocyte isolation

Eight-week-old C57/BL6 (\(n = 4\)) or BALB/c6 (\(n = 4\)) mice were killed with pentobarbitone. The spleen was carefully removed. Mouse splenic lymphocytes were isolated using a spleen cell dissociation kit (Miltenyi Biotec, Gladbach, Germany), according to the manufacturer’s instructions.

Human splenic lymphocytes were collected during splenectomy and isolated by using a spleen cell dissociation kit (Miltenyi Biotec). The isolated splenic lymphocytes were kept in the –80°C freezer for further experiments. The hospital human ethical committee approved all procedures and protocols (human ethical approval #B2013-115(3)).

### 2.4 | Lymphocyte transfer

One week after total body irradiation, the 5/6Nx mice were prepared for lymphocyte transfer. Lymphocytes, freshly isolated from either C57/BL6 (\(n = 2\)) or BALB/c6 (\(n = 2\)) mice, were prepared in PBS and injected i.v. into recipient (irradiated) C57/BL6 mice (10\(^7\) cells per mouse). The same procedure was repeated after 1 week.

### 2.5 | Isolation of glomeruli

Mouse kidneys were collected after perfusion with ice-cold PBS containing 8 × 10\(^7\) Dynabead ferric beads M450 (Invitrogen, Carlsbad, MO, USA) for 20 min (2 ml·min\(^{-1}\)). The kidneys were further digested with collagenase A (Roche, Basel, Switzerland) at 37°C for 30 min and filtered through 100-μm cell strainers. Glomeruli containing Dynabeads were isolated using a magnetic particle concentrator (Invitrogen). The isolated glomeruli were extracted with TRIZOL and stored at –80°C for further experiments.

### 2.6 | Gene expression assay

The glomeruli of 2K and 5/6Nx mice were collected for mRNA expression measurement. Three remnant kidneys were pooled for one sample (control = 3, 5/6Nx = 9, in 3 individual measurements). Total RNA was extracted using TRIZOL (Sigma, St. Louis, CA, USA). Gene expression was measured using a mouse inflammatory response and autoimmunity PCR arrays and mouse innate and adaptive immune PCR arrays (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Gene expressions were normalized to the house-keeping gene GAPDH (Takemoto et al., 2002).

### 2.7 | Endothelial cell culture

Primary human aortic endothelial cells (HAECs) and primary human renal glomerular endothelial cells (HGECs) were purchased from ScienCell (San Diego, CA, USA). Since endothelial cells undergo phenotypic drift when removed from their native environment (Minami & Aird, 2005), both were cultured and passaged (from passage 4 to 6) in endothelial culture medium (ScienCell). Experiments were performed in DMEM with 1% FBS (ScienCell). After simulations, cells were harvested for western blotting. Antioxidants (final concentrations are shown: apocynin 0.1 mM or ascorbic acid 30 μM), JNK inhibitors (SP600125 1 μM, or c-Jun peptide 2 μM), or NF-κB inhibitors (IT901, 1 μM, or pristimerin, 0.5 nM) were added to the culture medium 1 h before the stimulation.

### 2.8 | Transmigration assay

Transmigration assay was performed in 12-well plates as described (Krankel, Kuschnerus, Madeddu, Luscher, & Landmesser, 2011). Human splenic lymphocytes were thawed and suspended in DMEM culture medium. After staining with Trypan Blue, lymphocytes (5 × 10\(^4\)) were activated with phytohaemagglutinin (PHA, 2 μg·ml\(^{-1}\))
gentle MACS Dissociation (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured endothelial cells were lysed in lysis buffer containing 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM Tris–HCl, 1 mM NaF, 1 mM DTT, 10 μg/mL of aprotinin, 1 μg/mL of leupeptin, 1 mM Na3VO4, 1 mM phenyl ethylmalonylurea fluoride, and 1% NP-40 (Cell Signaling). Samples were then centrifuged at 20,000 x g for 15 min at 4°C. Protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). 

After perfusing with ice-cold PBS for 20 min (2 ml min⁻¹), the kidneys were collected and digested in Hanks buffer with 10 mg/mL of collagenase IV (Sigma-Aldrich) and 200 U/mL of DNase I (Invitrogen) at 37°C during 20 min. Renal cortex samples were dissociated using a gentle MACS Dissociation (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

Preparation of single-cell suspensions from mouse kidney

After permeabilizing with Perm/Wash Buffer (BD Biosciences, San Jose, CA, USA), the cells were stained intracellularly with antibodies for 1 h at 4°C. The stained cells were analysed using a FACSScan (CytoFlex LX, Beckman Coulter; Brea, CA, USA) and FlowJo software (RRID:SCR_008520, Tree Star Inc.).

Flow cytometry

Renal single cells were washed with FACS buffer and then stained for lineage markers for 30 min at 4°C. After permeabilizing with Perm/Wash Buffer, the cells were stained intracellularly with antibodies for 1 h at 4°C. The stained cells were analysed using a FACSScan and FlowJo software.

Western blotting

The antibody-based procedures used in this study comply with the recommendations made by the British Journal of Pharmacology. The antibody-based procedures used in this study comply with the recommendations made by the British Journal of Pharmacology. Protein presence was determined by western blotting. Renal cortex samples were dissociated using a gentle MACS Dissociation (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

Cytokine measurements

Renal cortex samples were lysed in lysis buffer containing 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM Tris–HCl, 1 mM NaF, 1 mM DTT, 10 μg/mL of aprotinin, 1 μg/mL of leupeptin, 1 mM Na3VO4, 1 mM phenyl ethylmalonylurea fluoride, and 1% NP-40 (Cell Signaling). Samples were then centrifuged at 20,000 x g for 15 min at 4°C, and the supernatant was collected and stored at −80°C. Levels of cytokine in the lysates from renal cortex samples were measured by Lumines (M60009RDPD, Bio-Plex Pro Mouse Cytokine 23-plex, Bio-Rad) according to the manufacturer’s protocol.

Haematoxylin and eosin staining, immunohistochemistry, and fluorescent examination

Mouse kidneys were fixed with a 10% formalin solution during at least 24 h and embedded in paraffin. Antigen retrieval was carried out by incubating the samples with 0.3% H2O2 during 30 min and heating to boiling (microwave oven) in the citrate buffer during 10 min. The sections were blocked with 5% goat serum in Tris-buffered saline and then incubated overnight at 4°C with diluted primary antibodies (1:200). Next, the sections were incubated in Super-Picture

Creatinine measurement

Plasma samples were obtained by centrifugation (500 x g) during 5 min at 4°C and stored at −20°C. Plasma creatinine levels were measured using the QuantiChrom Creatinine Assay Kit (BioAssay System; Hayward, CA, USA).

Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology. The design in this study complies with the recommendations on experimental design in pharmacology.
2018). Data are presented as means ± SEM and n refers to the number of mice or the number of individual experiments in cell culture. After the Shapiro–Wilks normality test, unpaired t-test and one-way ANOVA by Bonferroni post hoc test were performed (GraphPad Prism, Ver# 5, RRID:SCR_002798, GraphPad Software, San Diego, CA, USA). Experiments with n = 3 were not subjected to statistical analysis. Differences were considered to be statistically significant when P was less than 0.05.

2.16 | Materials
Apocynin, SP600125, c-Jun peptide, IT901, and pristimerin were purchased from Tocris Bioscience (Abingdon, UK). Ascorbic acid, collagenase IV, DMSO, pentobarbitone, PHA, and TNF-α were bought from Sigma-Aldrich (St. Louis, MO, USA) and butorphanol from Cayman (Ann Arbor, MI, USA). Amoxicillin was supplied by RuiYang (Shandong, PRC). Recombinant human IFN-γ and TGF-β1 were bought from PeproTech (Rocky Hill, NJ, USA). Dynabead magnetic beads M450 were bought from Invitrogen. Anti-rabbit (RRID:AB_2313567) and anti-mouse (RRID:AB_10015289) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-rabbit IgG-FITC (RRID:AB_631744) and anti-mouse IgG-FITC (RRID:AB_631735) antibodies were bought from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-rat MHC I antibody (Abcam ab15681, RRID:AB_302030) detects mouse MHC Class I antigens of d and b haplotype; anti-rabbit MHC I antibody (Abcam ab110645, RRID:AB_10859600) detects human HLA B; anti-rat MHC II antibody (Abcam ab23990, RRID:AB_447796) detects I-A region of the mouse MHC; and anti-mouse MHC II antibody (Abcam ab55152, RRID:AB_94419) detects human MHC II β chain HLA-DPB1. The primary antibodies are listed in Table 1.

2.17 | Nomenclature of targets and ligands
Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (https://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Fabbro et al., 2019; Alexander, Kelly et al., 2019a, 2019b).

3 | RESULTS

3.1 | 5/6Nx mice exhibit increased protein presence of MHC molecules in the remnant kidney
Four weeks after nephrectomy, 5/6Nx mice had fewer glomeruli than the control group (Figure 1a,b). 5/6Nx mice exhibited higher mRNA expressions of NADPH oxidase subunits and uncoupling protein 3 in the remnant kidney than the 2K group (Figure S2). They also exhibited higher levels of proinflammatory cytokines and chemokines in the remnant kidney than the 2K group, including IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, CXCL1, CCL2, CCL3 and CCL5 (Figures 1c and S3).

Isolated glomeruli of the 5/6Nx group exhibited increased mRNA expression of MHC, CD80, and CD86 (Figure S4). Immunohistochemical staining revealed that the MHC Class I (MHC I), MHC Class I chain-related protein A (MICA), and MHC Class II (MHC II) proteins were mainly present in glomeruli (Figure 1d,f,h). Immunofluorescence staining showed that the expression of MHC I, MICA, and MHC II molecules were colocalized with CD31+ endothelial cells (Figures 1e,g,i and S5–S8).

3.2 | The increased protein presence of MHC molecules in glomerular endothelial cells of 5/6Nx mice promotes lymphocyte movement
To understand the importance of the MHC complex up-regulation in glomerular endothelial cells, mice were transferred with splenic lymphocytes from either C57/BL6 or BALB/c mice, after total body irradiation (Figure 2a). Full body irradiation alone reduced serum creatinine levels in the 5/6Nx, but not in the 2K group. Lymphocytes injected from BALB/c, but not from C57/BL6 mice, significantly increased the serum creatinine level, when compared with 5/6Nx mice subjected to irradiation alone (Figure 2b).

To study the involvement of T lymphocytes in the remnant kidney after the irradiation, CD3+ T lymphocytes and its subgroups were analysed with FACS assays. Four weeks after the surgery, the T lymphocyte count (including CD3−, CD3+/CD4+, CD3+/CD8+, and CD3−/CD4+/CD25+/Foxp3+ subgroups) in the kidney was comparable, in the 2K and 5/6Nx groups. In line with increased levels of cytokines in the remnant kidneys, Th1 (CD3+/TNF-α+ and CD3+/IFN-γ+), Th2 (CD3+/IL-5+ and CD3+/IL-13+), and Th17 (CD3+/IL-17+) lymphocytes were significantly increased in the 5/6Nx groups (Figure S9). Full body irradiation significantly reduced CD3+ cells in the kidney. Lymphocyte transfer, from either C57/BL6 or BALB/c, restored CD3+ cells in the kidney. The T lymphocyte count, for CD3+/CD4+, CD3+/CD8+, and CD3−/CD4+/CD25+/Foxp3+ subgroups, was comparable in the transfer groups (Figure 2c).

Haematoxylin and eosin (H&E) staining revealed that irradiation followed by C57/BL6 lymphocyte transfer did not induce noticeable morphological changes in the glomeruli. However, lymphocytes transfer from BALB/c induced glomerular shrinkage and leakage in the glomerular capsules (Figure 2d).

To study the involvement of glomerular endothelial cells in the MHC complex up-regulation, CD31+ endothelial cells (Aird, 2007; Pusztazseri, Seelentag, & Bosman, 2006) and CD31+ cells in the kidney were analysed using FACS assays. The CD31+ cell count was comparable in the kidneys of all groups (Figure 3a).

The presence of MHC Class II molecules on the surface of CD31+ cells was significantly increased in 5/6Nx mice when compared with those in the 2K group. Mice in the irradiation group, as well as in the lymphocyte transfer groups (both from C57/BL6 and BALB/c),
| Antibody      | Company                      | Catalogue and RRID         | Application          |
|--------------|------------------------------|----------------------------|----------------------|
| APC Cy7 CD3  | BD Biosciences (San Jose, CA, USA) | 561042 RRID:AB_2034003    | FACS, Cell surface (1:100) |
| PE Cy7 CD3   | BD Biosciences (San Jose, CA, USA) | 552774 RRID:AB_394460     | FACS, Cell surface (1:100) |
| FITC CD4     | BD Biosciences (San Jose, CA, USA) | 557307 RRID:AB_396633     | FACS, Cell surface (1:100) |
| PerCP CD8    | BD Biosciences (San Jose, CA, USA) | 553036 RRID:AB_394573     | FACS, Cell surface (1:100) |
| PE CD25      | BD Biosciences (San Jose, CA, USA) | 553075 RRID:AB_394605     | FACS, Cell surface (1:100) |
| Alexa Fluor@700 Foxp3 | BD Biosciences (San Jose, CA, USA) | 560401 RRID:AB_1645201   | FACS, Intracellular (1:100) |
| PE IL-5      | BD Biosciences (San Jose, CA, USA) | 562049 RRID:AB_10894193   | FACS, Intracellular (1:100) |
| PE Cy7 IL13  | Invitrogen (Carlsbad, MO, USA)   | 25-7133-80 RRID:AB_2573529 | FACS, Intracellular (1:100) |
| BV421 IL-17  | BD Biosciences (San Jose, CA, USA) | 563354 RRID:AB_2687547    | FACS, Intracellular (1:100) |
| BV711 IFN-γ  | BD Biosciences (San Jose, CA, USA) | 564336 RRID:AB_2738752    | FACS, Intracellular (1:100) |
| APC TNF-α    | BD Biosciences (San Jose, CA, USA) | 561062 RRID:AB_2034022    | FACS, Intracellular (1:100) |
| APC CD31     | BD Biosciences (San Jose, CA, USA) | 551262 RRID:AB_398497     | IF (1:20) FACS, Cell surface (1:100) |
| BV421 CD31   | BD Biosciences (San Jose, CA, USA) | 562939 RRID:AB_2665476    | FACS, Cell surface (1:100) |
| FITC H-2K(d) | BD Biosciences (San Jose, CA, USA) | 562003 RRID:AB_10896664   | FACS, Cell surface (1:100) |
| PerCP-Cy I-A/I-E | BD Biosciences (San Jose, CA, USA) | 562363 RRID:AB_11153297   | FACS, Cell surface (1:100) |
| PE CD86      | BD Biosciences (San Jose, CA, USA) | 561963 RRID:AB_10896971   | FACS, Cell surface (1:100) |
| FITC CD80    | BD Biosciences (San Jose, CA, USA) | 561954 RRID:AB_10896321   | FACS, Cell surface (1:100) |
| BV510 CD45   | BD Biosciences (San Jose, CA, USA) | 563891 RRID:AB_2734134    | FACS, Cell surface (1:100) |
| CIITA        | Abcam (Cambridge, UK)          | ab117598 RRID:AB_10902499 | WB, 123 kDa (1:1,000) |
| MHC I        | Abcam (Cambridge, UK)          | ab15681 RRID:AB_302030    | IF (1:50) IHC (1:200) |
| MHC I + HLA B| Abcam (Cambridge, UK)          | ab110645 RRID:AB_10859600 | WB, 40 kDa (1:1,000) |
| MHC Class II | Abcam (Cambridge, UK)          | ab55152 RRID:AB_944199    | IF (1:50) IHC (1:200) |
| MICA         | Abcam (Cambridge, UK)          | ab62540 RRID:AB_956135    | IF (1:50) IHC (1:200) |

**TABLE 1** Antibody information
exhibited a comparably higher level of MHC II molecules, as 5/6Nx mice did (Figure 3b,d, upper panel). The presence of MHC Class II molecules on the surface of CD31^- cells was comparable in 2K and 5/6Nx mice. Mice in the BALB/c transfer group had significantly higher levels of MHC II expression in their CD31^- cells (Figure 3c,e, upper panel). The presence of CD80 molecules on the surface of CD31+ cells was increased significantly in 5/6Nx mice. Mice in the irradiation group, as well as those transferred with lymphocytes from

| Antibody                  | Company                                      | Catalogue and RRID | Application          |
|---------------------------|----------------------------------------------|--------------------|----------------------|
| Phospho-SAPK/JNK          | Cell Signaling Technology (Danvers, MA, USA) | 4668               | WB, 54 and 46 kDa (1:1,000) |
|                           |                                               | RRID:AB_823588     |                      |
| SAPK/JNK                  | Cell Signaling Technology (Danvers, MA, USA) | 9252               | WB, 54 and 46 kDa (1:1,000) |
|                           |                                               | RRID:AB_2250373    |                      |
| Phospho-NF-κB p65 (Ser536)| Cell Signaling Technology (Danvers, MA, USA) | 3033               | WB, 65 kDa (1:1,000)  |
|                           |                                               | RRID:AB_331284     |                      |
| NF-κB p65                 | Cell Signaling Technology (Danvers, MA, USA) | 8242               | WB, 65 kDa (1:1,000)  |
|                           |                                               | RRID:AB_10859369   |                      |
| GAPDH                     | Millipore (Burlington, MA, USA)               | MAB374             | WB, 38 kDa (1:2,000)  |
|                           |                                               | RRID:AB_2107445    |                      |

**FIGURE 1** Mice subjected to 5/6 nephrectomy exhibit chronic inflammation in the cortex of remnant kidneys and increase MHC protein in glomeruli, 4 weeks after the surgery. (a) Isolated glomeruli under microscopy (left, low resolution; right, high resolution). (b) Glomeruli count in the kidney of 2K and 5/6Nx mice. n = 9; *P < 0.05, significantly different from the control 2K mice. (c) Levels of inflammatory cytokines in the kidney of 2K and 5/6Nx mice. n = 5; *P < 0.05, significantly different from the control 2K mice. (d-i) Presence of MHC molecule complex in glomeruli of 2K and 5/6Nx. Presence of MHC I (d), MICA (f), and MHC II (h) by immunohistochemistry staining. Presence of MHC I (e), MICA (g), MHC II (i), and CD31+ endothelial cells by immunofluorescent examination.
C57/BL6 mice, had a comparably higher expression of CD80 molecule (Figure 3b,d, middle panel). Lymphocyte transfer from BALB/c mice did not change the expression of CD80 in CD31+ endothelial cells. The presence of CD80 in CD31− cells was comparable in the four groups (Figure 3c,e, middle panel). The presence of CD86 molecule on the surface of CD31+ cells was significantly increased in 5/6Nx mice but was comparable in CD31− cells of the four groups (Figure 3c,e, lower panel).

3.3 | TNF-α and IFN-γ up-regulate protein presence of MHC complexes in cultured HGECs

To explore the mechanisms underlying the MHC up-regulation in glomerular endothelial cells, both primary HAECs and HGECs were cultured in vitro. TNF-α (3 ng·ml⁻¹) and IFN-γ (10 ng·ml⁻¹) were used as stimuli based on their increased levels in the cortex of 5/6Nx mice (Figure 1e). TGF-β1 (5 ng·ml⁻¹) was used as a negative regulator as TGF-β1 counteracts IFN-γ-mediated responses (Banu & Meyers, 1999).

Under basal conditions, HAECs exhibited a higher level of MHC I, but not of MICA or MHC II. Stimulation during 72 h with TNF-α, TGF-β1, or IFN-γ alone did not change expressions of the MHC proteins. Combined stimuli did not increase MHC expression either (Figure 4a, left panel, and Figure S10).

Under basal conditions, HGECs exhibited lower levels of MHC I, MICA, or MHC II. After 72 h, TNF-α significantly increased MICA and MHC II, IFN-γ significantly increased MHC I, MICA, and MHC II and TGF-β1 did not change levels of any MHC protein. Combined stimulation did not have additional effects (Figure 4a, right panel, and Figure 4b).

3.4 | CIITA and MAPK–JNK play an important role in the regulation of MHC complexes in cultured HGECs

The antioxidants apocynin and ascorbic acid were used, as oxidative enzymes were up-regulated in the renal cortex of 5/6Nx mice (Figure S3). NF-κB inhibitors, IT901 and pristimerin, were also tested.
as NF-κB plays an important role in the regulation of MHC I molecules (Girdlestone, 2000). Likewise, the effect of JNK inhibitors (SP600125 or c-Jun peptide) was determined as MAPK–JNK is more involved in the regulation of antigen presentation, including MHC Class II trans-activator (CIITA) and genes encoding MHC Class II proteins, compared with other MAPKs (Valledor et al., 2008).

After 24 h, IFN-γ significantly increased CIITA protein. JNK inhibitors and NF-κB inhibitors, but not antioxidants, inhibited the increased presence of CIITA protein (Figure 5a,b, left panel). Western blotting with antibodies against phosphorylated JNK at specific amino acid residues revealed that IFN-γ increased the phosphorylation of p54 and p46 of JNK. IFN-γ did not exert significant effects on total JNK protein levels. The antioxidants apocynin and ascorbic acid did not reduce phosphorylated levels or total protein of JNK. Inhibitors of JNK and NF-κB reduced the phosphorylated level of JNK (Figure 5a,b, left panel).

Western blotting with antibodies against phosphorylated p65–NF-κB complex at specific amino acid residues revealed that IFN-γ increased the phosphorylation of p65 of NF-κB. IFN-γ did not exert significant effects on total p65–NF-κB protein levels. Inhibitors of JNK and NF-κB reduced the phosphorylated level of p65–NF-κB in HGECs (Figure 5a,b, left panel).

After 72 h, antioxidants did not reduce the IFN-γ-induced up-regulation of MHC I, MICA, or MHC II. JNK (SP600125 or c-Jun peptide) and NF-κB (IT901 or pristimerin) inhibitors significantly reduced the IFN-γ-induced up-regulation of MHC I, MICA, and MHC II (Figure 5a,b, left panel).

After 24-h stimulation with TNF-α, CIITA protein was significantly increased (Figure 5a,b, right panel). JNK inhibitors and NF-κB inhibitors, but not antioxidants, inhibited this up-regulation of CIITA protein.
Western blotting with antibodies against phosphorylated JNK at specific amino acid residues revealed that incubation of HGEC with TNF-α increased the phosphorylation of p54, but not those of p46 or JNK proteins. TNF-α did not exert significant effects on total JNK protein levels. Neither antioxidants nor inhibitors of NF-κB reduced the phosphorylated level or total protein presence of JNK (Figure 5a,b, right panel).

Western blotting with antibodies against phosphorylated p65–NF-κB complex revealed that TNF-α increased the phosphorylated level of p65 of NF-κB complex. TNF-α did not exert significant effects on total p65–NF-κB protein levels. Antioxidants and JNK inhibitors reduced the phosphorylated level of p65–NF-κB (Figure 5a,b, right panel).

After 72 h, antioxidants reduced the TNF-α-induced up-regulation of MHC II, but not that of MICA. By contrast, JNK inhibitors (SP600125 or c-Jun peptide) significantly reduced the TNF-α-induced up-regulation of both MICA and MHC II molecules. NF-κB inhibitors (IT901 or pristimerin) significantly reduced the TNF-α-induced up-regulation of MICA, but not MHC II molecule (Figure 5a,b, right panel).

After 3-day stimulations, either with IFN-γ or TNF-α, there were few floating cells in the HGEC culture medium. However, culture medium alone, either freshly prepared or experimental medium during 72-h incubation, did not stimulate lymphocytes migration (data not shown). Splenic lymphocytes in the transwell chamber, in the presence or absence of PHA, did not migrate to HGEC with the freshly changed medium (Figure 6b,c). Splenic lymphocytes in the transwell chamber, in the presence or absence of PHA, migrated to HGEC stimulated with IFN-γ and TNF-α for 72 h (Figure 6d,e).

Inhibitors of JNK and NF-κB, but not antioxidants, significantly reduced the lymphocyte migration stimulated by IFN-γ (Figure 6f) and that induced by TNF-α, was reduced by inhibitors of JNK, but not antioxidants or inhibitors of NF-κB (Figure 6g).

### 3.5 The increased protein presence of MHC complexes in HGEC promotes human lymphocyte movement

To understand the importance of the MHC complex up-regulation for lymphocyte migration, experiments of lymphocyte migration were designed (Figure 6a).

### 3.6 Inhibition of MAPK–JNK down-regulates MHC in 5/6Nx mice

In 5/6Nx mice, JNK inhibitors (SP600125 or c-Jun peptide) slightly, but significantly reduced serum creatinine levels, compared with untreated 5/6Nx mice (Figure 7a). The JNK inhibitors significantly reduced MHC II expression on the surface of CD31+, but not in CD31− cells. They did not change CD80 or CD86 expression in the 5/6Nx (Figure 7b–d). JNK inhibitors significantly reduced TNF-α and IL-17, but not IFN-γ, IL-5, or IL-13 expression in CD3+ lymphocytes (Figure 7e).
Renal mass removal in an animal provides a model mimicking chronic renal failure, in which glomeruli are challenged continuously with hyper-fluid filtration and chronic inflammation, leading to a vicious cycle of glomerulosclerosis (Brenner, Meyer, & Hostetter, 1982; Jin et al., 2017; Olson & Heptinstall, 1988). The present study reports that following nephrectomy, increased levels of proinflammatory cytokines promote up-regulation of MHC in glomerular endothelial cells. The enhanced presence of MHC complexes further triggers immune responses, which can be reversed by JNK inhibition.

Inflammatory responses play a key role in sabotaging endothelial function (Ankeny, Hinds, & Nerem, 2013; Aoki, Fukuda, Nishimura, Nozaki, & Narumiya, 2014; Davies, 2009; Siasos et al., 2018). In the present study, levels of IFN-γ and TNF-α in the remnant kidney are lower than those reported in animal models of acute renal failure, including renal ischaemia–reperfusion injury (Zhang et al., 2018) and cisplatin nephrotoxicity models (Ramesh & Reeves, 2002), confirming

FIGURE 5  IFN-γ (a) and TNF-α (b) regulate MHC protein differently. (a) (left) Representative western blots of MHC I, MICA, MHC II, phosphorylated p65 of NF-κB, total p65 NF-κB protein, phosphorylated MAPK–JNK, total MAPK–JNK, and GAPDH protein in HGECs when stimulated with IFN-γ (10 ng ml⁻¹). (a) (right) Densitometric quantification of MHC I, MICA, MHC II, phosphorylated p65 of NF-κB, total p65 NF-κB protein, phosphorylated MAPK–JNK, and total MAPK–JNK in HGEC when stimulated with IFN-γ, n = 5; *P < 0.05, significantly different from cells under control condition. (b) (left) Representative western blots of MHC I, MICA, MHC II, phosphorylated p65 of NF-κB, total p65 NF-κB protein, phosphorylated MAPK–JNK, total MAPK–JNK, and GAPDH protein in HGECs when stimulated with TNF-α (3 ng ml⁻¹). (b) (right) Densitometric quantification of MHC I, MICA, MHC II, phosphorylated p65 of NF-κB, total p65 NF-κB protein, phosphorylated MAPK–JNK, and total MAPK–JNK in HGEC when stimulated with TNF-α, n = 5; *P < 0.05, significantly different from cells under control conditions

4 | DISCUSSION
that chronic inflammatory responses occur in the early stage of chronic renal failure. Both IFN-γ and TNF-α are critical in the regulation of MHC complexes (Konieczny et al., 1998; Taflin et al., 2011; Wedgwood et al., 1988), indicating that these two proinflammatory cytokines also play a role in the immunology of allografts. TGF-β1 inhibits IFN-γ-mediated microglial activation (Zhou, Zoller, Krieglstein, & Spittau, 2015) and down-regulates inducible MHC protein in renal tubular epithelial cells (Banu & Meyers, 1999). However, the present experiments showed that 5/6 nephrectomy did not increase TGF-β1 expression and that stimulation with TGF-β1 did not alter MHC expression in cultured endothelial cells. Therefore, the present study focused on the mechanisms underlying MHC induction in glomerular endothelial cells in the presence of IFN-γ or TNF-α.

IFN-γ and TNF-α up-regulated MHC molecules in HGECS (but not in HAECs), resulting in lymphocyte activation and migration, thus suggesting that renal microvascular endothelial cells have a high potential of presenting antigens and possess immunogenicity to allogeneic lymphocytes. The up-regulation of MHC proteins is pivotal for the adaptive immune system, especially in transplant immunology. MHC Class I proteins are constitutively expressed on most nucleated cells. MICA increases antigen diversity and plays a role in chronic transplant rejection (Zhang et al., 2011; Zwirner, Dole, & Sustny, 1999). MHC Class II molecules are mainly expressed by cells of immune lineages (Petranyi, 1995; Raval, Puri, Rath, & Saxena, 1998) and are important in allograft rejection (van der Stoep, Quinten, & van den Elsen, 2002). In the present study, enhanced expression of MHC proteins, including MHC I, MICA, and MHC II, were confirmed both in vivo and in vitro in glomerular endothelial cells. In cultured glomerular endothelial cells, MHC expression was lower under quiescent conditions but were increased when stimulated with TNF-α or IFN-γ (Wedgwood et al., 1988). These observations confirm that renal microvascular endothelial cells require basal physiological
FIGURE 7  Inhibition of MAPK–JNK partly restores renal function in 5/6Nx mice. (a) Serum levels of creatinine. (b) Quantification of MHC II⁺ (upper), CD80⁺ (middle), and CD86⁺ (lower) signals in the CD31⁺ group in 5/6Nx mice treated with JNK inhibitors. (c) Quantification of MHC II⁺ (upper), CD80⁺ (middle), and CD86⁺ (lower) signals in the CD31⁻ group in 5/6Nx mice treated with JNK inhibitors. (d) The presence of MHC II⁺, CD80⁺, and CD86⁺ signals in the CD31⁺ group measured by FACS in 5/6Nx mice treated with JNK inhibitors. (e) The presence of MHC II⁺, CD80⁺, and CD86⁺ signals in the CD31⁻ group by FACS in 5/6Nx mice treated with JNK inhibitors. (f) Quantification of TNF-α, IFN-γ, IL-5, IL-13, and IL-17 in CD3⁺ lymphocytes. n = 9–10; *P < 0.05, significantly different from control mice (2K); #P < 0.05, significantly different from 5/6Nx mice.
concentrations of cytokines to maintain the presence of MHC protein (Muczynski, Ekle, Coder, & Anderson, 2003).

CIITA is essential in regulating MHC proteins for both Class I (Goblin, Peijnenburg, Keijzers, & van den Elsen, 1997; Martin et al., 1997) and Class II (Chang, Guerder, Hong, van Ewijk, & Flavell, 1999; Steimle et al., 1993; van der Stoep & Mach, 1994). Mutation of CIITA down-regulates MHC II protein presence (Steimle et al., 1993; Wong et al., 2014), and overexpressing it restores MHC II expression in Class II-depleted cells (Chang, Fontes, Peterlin, & Flavell, 1994; Steimle et al., 1993; van der Stoep et al., 2002). In the present experiment, both IFN-γ and TNF-α increased CIITA and MHC proteins, supporting the concept that CIITA is the master regulator of the MHC protein family. The CIITA-associated transcription factors include RFX, cAMP response element modulator, nuclear transcription factor Y, histone deacetylase, PR domain zinc finger protein 1 (Masternak et al., 2000), STAT, NF-κB complex, and Jun (Wong et al., 2014). The involvement of transcription factors varies depending on the stimulating agonists applied, the experimental models examined, or the cultured cells studied. Of note, MHC I genes have a distinct region bound by Rel/NF-κB factors. During challenges with TNF-α or IFN-γ, CIITA works together with RelA to promote MHC I transcription (Girdlestone, 2000). In the present study, both TNF-α and IFN-γ increased the phosphorylated level of NF-κB p65 (also known as RelA). In the presence of TNF-α, NF-κB inhibitors reduced the levels of MICA, but not those of MHC II, whereas, in the presence of IFN-γ, NF-κB inhibitors reduced both MICA and MHC I proteins. These data confirm the active role of RelA in the regulation of the MHC family.

MAPK is a stress-induced kinase, exerting multiple effects on cell proliferation, inflammation, and migration. Activation of MAPK and up-regulation of MHC II molecules are parallel in all cases (Martin et al., 2007). Both MAPK–ERK and MAPK–JNK take part in the regulation of CIITA transcription in melanoma (Martins et al., 2007). In marrow-derived dendritic cells and macrophages, MAPK–ERK and MAPK–p38, but not MAPK–JNK, inhibit CIITA presence by decreasing histone acetylation of the CIITA promoter (Yao et al., 2006). MAPK–JNK, but not MAPK–p38, participates mainly in IFN-γ-induced antigen presentation, including CIITA and genes encoding MHC Class II molecules in macrophages (Valledor et al., 2008) and brain endothelial cells (Adamski & Benveniste, 2005). In the present study, IFN-γ and TNF-α activated MAPK–JNK and inhibition of MAPK–JNK reduced protein MHC expression in glomerular endothelial cells in vivo and in vitro. These data confirm the critical role of MAPK–JNK in regulating the MHC family. Furthermore, inhibition of JNK blunted NF-κB activation in cells stimulated with IFN-γ or TNF-α, indicating that MAPK–JNK signalling is upstream of NF-κB activation. However, inhibition of NF-κB reduced the phosphorylation of JNK in cells stimulated with IFN-γ, but not TNF-α, suggesting that IFN-γ and TNF-α trigger different signalling pathways in the regulation of MHC molecules. JNK inhibitors reduced TNF-α and IL-17 expressions in infiltrating T lymphocytes, suggesting that MAPK–JNK is also involved in signal integration during costimulation of T lymphocytes (Li, Whaley, Mondino, & Mueller, 1996; Su et al., 1994), including Th17 cells (May et al., 2019). The inactivation of Th1 and Th17 cells following inhibition of JNK signalling is most likely due to the direct inhibitory effects on lymphocytes, lower MHC complex expression on the glomerular endothelial cells or both.

Furthermore, the present study demonstrated that mice subjected to total body irradiation had fewer lymphocytes infiltrated in their remnant kidney, suggesting that total body irradiation down-regulates the endogenous immune system and attenuates renal dysfunction. Exogenous lymphocytes, from either homogenetic or heterogenetic background mice, homed to the remnant kidney. Increased levels of chemokines in the remnant kidney suggest that some chemotactic substances are produced in 5/6Nx mice. In addition, lymphocytes transferred from heterogenetic mice, but not those of homogenetic mice, increased serum creatinine levels in 5/6Nx mice, implying that the allograft immune responses led to further deterioration in kidney function.

In the present study, the remnant kidney had increased levels of mRNA for NADPH oxidase subunits and uncoupling protein 3, indicating that oxidative stress is enhanced. However, antioxidants failed to reduce TNF-α and IFN-γ-induced MHC up-regulations in vitro, suggesting that the increased oxidative stress probably plays a more critical role in inflammatory responses (shown as increased levels of cytokines), rather than in the MHC regulations.

Species variation provides serious problems in the interpretation of rodent results. Murine vascular endothelium constitutively expresses MHC I and CD80 but is unable to induce acute graft rejection via CD4+ direct allorecognition (Kreisel et al., 2004). Human endothelial cells express leukocyte function antigen-3, but not CD80, and can induce allorecognition of CD8+CD4+ cells (Hughes, Savage, & Poer, 1990). The up-regulation of MHC activates lymphocytes and promotes their movement both in vivo and in vitro experiments, demonstrating that the up-regulation of MHC complex on endothelial cells leading to lymphocyte activation is a general mechanism in both human and rodents.

Glomeruli and their endothelial cells under pathological conditions are challenged with inflammation as well as chronic haemodynamic changes, resulting in a reduced number of functional glomeruli and progressive loss of renal function in a vicious cycle. In the present study, overloaded glomerular endothelial cells present up-regulation of MHC complexes, confirming the critical role of glomerular endothelial cells in the circulation and immune system. In addition to well-studied macrovascular endothelial dysfunctions, renal microvascular endothelial cells have a great potential to be antigen-presenting cells, demonstrating that apart from myeloid cells and lymphocytes, structural cells including those of the endothelium are critical regulators in immune responses (Krausgruber et al., 2020). Of note, the protective effects of JNK inhibition in both endothelial cells and infiltrating lymphocytes observed here provide valuable information for the treatment of chronic nephritis and renal transplantation.

In summary, the data in the present study support the clinical observation that endothelial cells are the first target in graft rejection. In the present study, elevated levels of proinflammatory cytokines...
take part in the up-regulation of MHCs in glomerular endothelial cells through activation of JNK. The regulation of the CIITA gene by MAPK–JNK may be an important factor in a better understanding of the physio-pathological expression and dysregulation of MHC complexes.

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AUTHOR CONTRIBUTIONS
D.Z., Q.T., and B.Y. performed the experiments and analysed the data; M.M., W.L., and J.L. helped the animal experiments. D.Z., T.Z., S.L., Y.Z., and Y.S. wrote the manuscript; Y.Z. and Y.S. designed the experiments; P.M.V., S.L., Y.Z., and Y.S. gave final content approval; and all authors read and edited/revised the manuscript.

CONFICT OF INTEREST
The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting & Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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