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RESEARCH ARTICLE

Aspirin alleviates the symptoms of immunoglobulin A nephropathy *via* suppressing platelets-mediated non-canonical NF-κB activation in B cells

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Abstract

***Purpose*:** Antiplatelet aggregation drugs, such as aspirin, can alleviate pathological renal damage in immunoglobulin A (IgA) nephropathy, although the precise mechanism is unclear.
***Methods*:** The serum levels of platelet factor 4 (PF4), IgA, and platelet-activating factor (PAF) were assessed by enzyme-linked immunosorbent assay in IgA nephropathy patients and TANK-binding kinase 1 (TBK1)$^{-/-}$ tumor necrosis factor (TNF)$^{-/-}$ mice. The deposition of IgA in glomeruli was detected by immunofluorescence. Phorbol-12-myristate-13-acetate (PMA) induced platelets activation was examined by the cell counting kit 8 assay. B cells were further stimulated with lipopolysaccharides (LPS) or plus platelets supernatant, or combined with nuclear factor kappa-B (NF-κB) inducing kinase (NIK) inhibitor, NIK SMI1.
***Results*:** Increased serum IgA and proportion of activated platelets were observed in IgA nephropathy patients. TBK1$^{-/-}$TNF$^{-/-}$ mice had significant increased urinary protein and serum creatinine, and IgA deposition in glomeruli. Up-regulated serum PF4 and PAF were observed in both the IgA nephropathy patients and TBK1$^{-/-}$TNF$^{-/-}$ mice. Aspirin suppressed the deposition of IgA in glomeruli of TBK1$^{-/-}$TNF$^{-/-}$ mice with down-regulated platelets activation. Platelets supernatant could promote the proliferation of B cells with up-regulated IgA and sCD40L secretion and up-regulated P52 and RelB expression, which could be inhibited by NIK SMI1 administration.
***Conclusion*:** TBK1$^{-/-}$TNF$^{-/-}$ mice demonstrate IgA nephropathy phenotype, which could be alleviated by aspirin administration *via* inhibiting platelets induced non-canonical NF-κB activation mediated IgA production in B cells.

Keywords: *aspirin*; *IgA nephropathy*; *NF-κB*; *B cells*

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Immunoglobulin A (IgA) nephropathy can progress to end-stage (15–20%, within 10 years; 30–40%, within 20 years) (1, 2). Currently, no IgA nephropathy-specific therapy is recommended, and patients are managed with supportive therapy, such as renin-angiotensin system blockade and renal function maintenance (3).

Antiplatelet aggregation drugs can reduce pathological renal damage, delay the progress of nephritis, and protect renal function, which has been widely utilized in Chinese and Japanese populations (4, 5). While the relevant mechanism involved in the action of the antiplatelet drug, aspirin, on IgA nephropathy is not clear.

Elevated spontaneous IgA synthesis is observed in IgA nephropathy patients, indicating IgA-specific B cell hyperactivity (6). It is worth noting that the ratio of platelet-to-lymphocyte is an independent prognostic factor for renal survival in patients with IgA nephropathy (7). The number of activated platelets detected in the urinary sediments correlates with the IgA nephropathy stage (8). These data indicate that the interaction between platelet and B cells is involved in the development and progress of IgA nephropathy.

In this work, a new IgA nephropathy model was utilized to decipher the action of aspirin in IgA nephropathy, and the interaction between platelets and B cells is investigated. We find that aspirin alleviates the symptoms of IgA nephropathy *via* suppressing platelet-mediated non-canonical nuclear factor kappa-B (NF-κB) activation in B cells.

Methods and materials

Patient samples

Kidney biopsy-proven IgA nephropathy patients (61 cases, 16–80 years old) with impaired kidney function were enrolled in this investigation. Healthy volunteers (67 cases, 18–55 years old) without kidney damage or systemic diseases were involved in this study. The Ethics Committee of Faculty of Science Tshwane University of Technology approved all the research, and the written census was signed.

Platelets isolation, activation, and B cells co-culture

Ficoll-Hypaque density gradient (Millipore, Billerica, MA, USA) centrifugation was utilized to get human and mice platelets from venous blood, which were pelleted by centrifugation at 2,000 × g for 20 min and further incubated with phorbol-12-myristate-13-acetate (PMA, 10 ng/mL) to induce the activation. Anti-CD19-conjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to purify the B cells from splenocytes, which were then cultured for 5 days in Dulbecco's Modified Eagle Medium medium with 1 μg/mL lipopolysaccharides (LPS, Sigma, St. Louis, MO, USA) and 2 ng/mL transforming growth factor-beta (TGF-β, Peprotech, Bedford, MA, USA) or platelets supernatant.

Mice

All protocols were approved by the Institutional Animal Care and Use Committee of Faculty of Science Tshwane University of Technology. Aspirin (200 mg/kg, solubilized in 0.1% methylcellulose) was administered *via* oral gavage three times each week.

Cell-Counting Kit-8 (CCK-8) assay

CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was utilized to detect cell viability. 5×10^5 cells were cultured in 96-well plates for 24 h, then incubated with CCK-8 solution for another 2 h at 37°C. The absorbance at 450 nm was recorded.

Immunoblot assay

The lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, which were incubated with the primary antibodies specific for p100/p52, Rel B, and LaminB, and further incubated with peroxidase-conjugated secondary antibody. All antibodies were provided by Santa Cruz (Dallas, TX, USA).

Enzyme-linked immunosorbent assay

The levels of IgA, TGF-β, B cell activating factor (BAFF), platelet factor 4 (PF4), platelet-activating factor (PAF), and soluble CD40L (sCD40L) were measured with enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA). Urine Protein ELISA Kit (antibodies-online GmbH) and creatinine ELISA Kit (BioVision, Milpitas, CA, USA) were utilized to detect the relevant protein in the urine and serum.

Immunofluorescence

Mice kidneys were rapidly frozen in and processed to produce 2 μm cryostat sections, which were fixed in cold acetone and stained overnight with fluorescein isothiocyanate (FITC)–labeled rat anti-mouse IgA (BD Biosciences, Franklin Lakes, NJ, USA).

Statistics

Statistics were performed using GraphPad Prism software. Statistical evaluations were performed by the Student's *t*-test, or one-way analysis of variance followed with a Tukey's post hoc test.

Results

Increased IgA and activated platelets in IgA nephropathy patients

B cells dysregulation may result in the development of IgA nephropathy, and TGF-β and BAFF are testified to regulate B cells responsiveness, IgA induction, and B-cell maturation (9, 10). In this investigation, increased IgA (Fig. 1a), TGF-β, and BAFF (Fig. 1b) could be observed in the serum of IgA nephropathy patients when compared with the healthy volunteers. Interestingly, compared with the healthy control group, patients with IgA nephropathy also showed increased platelet activation (Fig. 1c) and the up-regulated PAF and PF4 secretion (Fig. 1d). All of these data indicated that the dysregulation of B cells and platelet activation contributed to the development of IgA nephropathy.

TANK-binding kinase $1^{-/-}$tumor necrosis factor$^{-/-}$ mice displayed the symptoms of IgA nephropathy

Increased IgA and IgG deposits (Fig. 2a) were detected in the glomeruli of TANK-binding kinase $1^{-/-}$tumor necrosis factor$^{-/-}$ ($TBK1^{-/-}TNF^{-/-}$) mice. At the same time, increased urine protein and serum creatinine were detected in $TBK1^{-/-}TNF^{-/-}$ mice (Fig. 2b), which indicated the dysregulated glomerular filtration. It was worth noting that the proportion of activated platelets and the concentration of platelets activation associated with PF4 and PAF (Fig. 2c) were also up-regulated. All of these results indicated that $TBK1^{-/-}TNF^{-/-}$ mice showed the phenotype of IgA nephropathy.

Aspirin suppresses the deposition of IgA in glomeruli of $TBK1^{-/-}$ $TNF^{-/-}$ mice

Aspirin treatment could significantly inhibit the proportion of activated platelets (Fig. 3a) and PAF secretion in the serum (Fig. 3b) of $TBK1^{-/-}TNF^{-/-}$ mice. The deposition

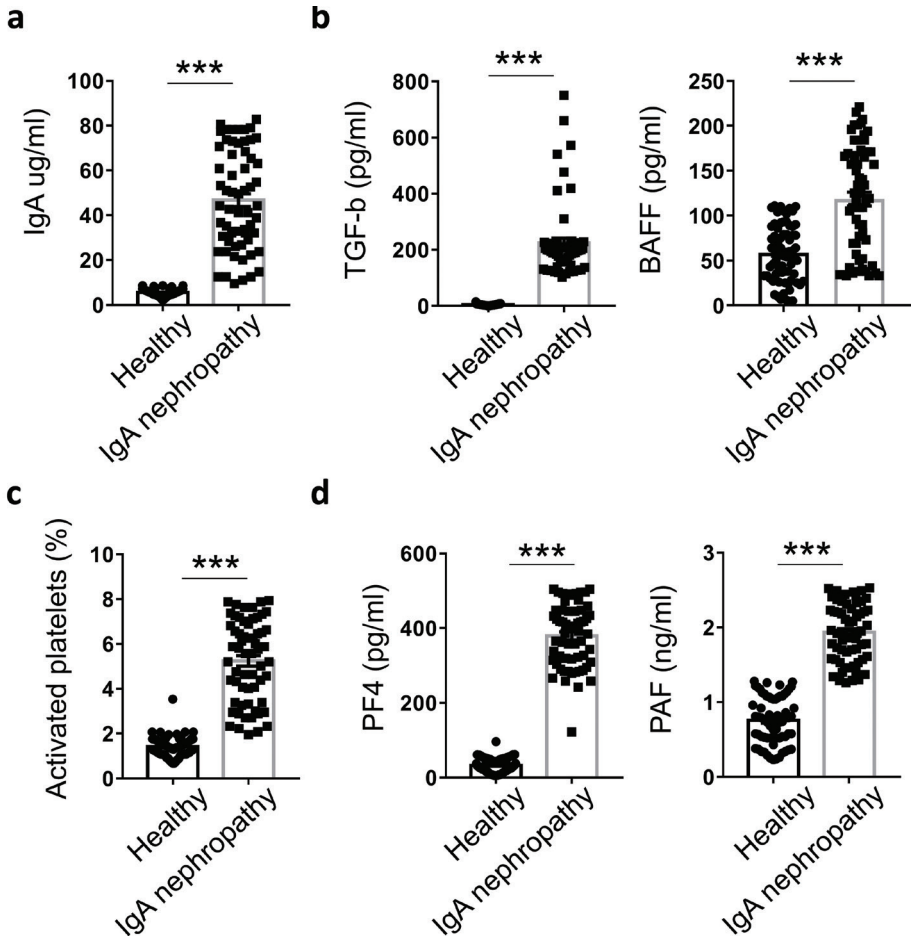

Fig. 1. Increased IgA and activated platelets were observed in the patients with IgA nephropathy. (a, b) The content of IgA (a), TGF-β and BAFF (b) from the serum of IgA nephropathy patients (n = 67) or healthy controls (n = 61) were assessed by ELISA. (c) Platelet activation induced with phorbol-12-myristate-13-acetate (PMA). (d) The protein levels of PF4 and PAF in the serum of IgA nephropathy patients or healthy controls were assessed by ELISA. The data were expressed as means ± standard error of mean (SEM). Results represent mean values of three independent experiments. ***, $P < 0.001$.

of IgA in the glomeruli of TBK1$^{-/-}$TNF$^{-/-}$ mice was significantly decreased after the administration of aspirin (Fig. 3c). All of these results demonstrated that aspirin could inhibit the activation of platelets and the relevant IgA deposition.

Platelets promote IgA production in B cells via non-canonical NF-κB pathway

In order to explore the interaction between platelets and B cells, the platelets supernatant was utilized to incubate B cells, which could significantly promote B cells proliferation induced by LPS (Fig. 4a, more than two folds). Interestingly, the platelets supernatant alone could neither up-regulate the proportion of activated B cells (Fig. 4a) nor promote IgA secretion (Fig. 4b). In contrast, the platelets supernatant could promote IgA secretion induced by LPS and TGF-β (Fig. 4b), indicating that platelets can only react to activated B cells. In the co-incubation system, we observed up-regulated content of sCD40L induced by the administration of the platelets supernatant (Fig. 4c), suggesting that sCD40L may mediate the interaction between platelets and B cells. IB results showed that the platelets supernatant promoted the activation of the non-classical NFκB signaling pathway in B cells with up-regulated p52 and RelB expression (Fig. 4d). NF-κB-inducing kinase (NIK) inhibitor, NIK SMI, can significantly down-regulate IgA secretion induced by the platelet supernatant (Fig. 4e). All of these results indicated that sCD40L might mediate the interaction between platelets and B cells, and the non-canonical NF-κB pathway contributed to the secretion of IgA in B cells induced by platelets.

Discussion

In this investigation, we find that TBK1$^{-/-}$TNF$^{-/-}$ mice show significant IgA deposits in the glomeruli and IgA secretion in the serum and altered glomerular filtration with the proteinuria and high creatinine, which could be utilized as

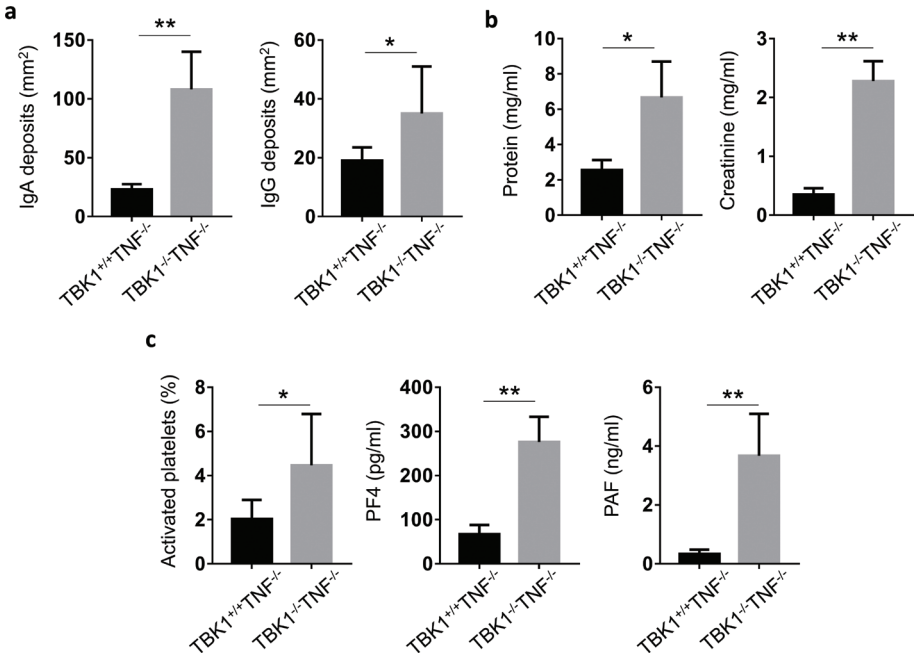

Fig. 2. Tbk1$^{-/-}$TNF$^{-/-}$ mice displayed the symptoms of IgA nephropathy. (a) The deposition of IgA and IgG in glomeruli of wild-type (WT) and TBK1$^{-/-}$TNF$^{-/-}$ mice were quantified. (b) Urinary protein and serum creatinine in WT and TBK1$^{-/-}$TNF$^{-/-}$ mice were measured by ELISA. (c) The protein levels of PF4 and PAF in the serum from WT and TBK1$^{-/-}$TNF$^{-/-}$ mice were assessed by ELISA. The data were expressed as means ± standard error of mean (SEM). Results represent the mean values of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IgA nephropathy model. Aspirin alleviates the symptoms of IgA nephropathy with down-regulated IgA deposits *via* inhibiting platelets-dependent non-canonical NF-κB pathway-mediated B cells proliferation and IgA production.

TBK1 phosphorylates NIK, which is subsequently degraded and negatively regulates IgA class switching (11). TBK1 knock-out mice are embryonic lethal, and TBK1 and TNF double knock mice can survive. In this investigation, we find that TBK1$^{-/-}$TNF$^{-/-}$ mice show IgA nephropathy-like phenotype, which can be further utilized to decipher the relevant mechanism.

Aggregated platelets may have controversial roles in releasing pro- or anti-inflammatory mediators dependent on the texture (12). Platelets are the primary purveyor of sCD40L, which can bind to CD40 located on peripheral B lymphocytes to induce the proliferation, antibody secretion, immunoglobulin class switching, and survival of memory B cells (13–16). On the other hand, CD40L can directly act on glomerular epithelial cells to modify glomerular permeability (17). In the clinic, elevated circulating sCD40L is associated with the severity of retinopathy in type 2 diabetes (18). It is worth noting that sCD40L can not only stabilize platelet thrombi to promote thrombosis but also induce β3 integrin tyrosine phosphorylation to activate platelet (19). All of these researches indicate that sCD40L-mediated mechanism may promote the pathology of IgA nephropathy.

On the other hand, the platelet activation related PF4 and PAF are up-regulated in the TBK1$^{-/-}$TNF$^{-/-}$ mice. PF4 is a chemokine derived from megakaryocytes or platelets, which can induce bone marrow B cell development and differentiation (20). PAF, a potent phospholipid, could modulate the early and late B cells activation events (21, 22). All of these results strengthen that the interaction between platelets and B cells might have a vital role in the development of IgA nephropathy.

As a family of transcription factors, NF-κB is identified initially to bind the enhancer of the immunoglobulin κ light-chain gene (23, 24). The non-canonical NF-κB pathway involves p100-sequestered NF-κB2 p52 and RelB activation, which is critical for B cell development, mature B-cell survival, and function (25, 26). All in all, this investigation indicates that TBK1$^{-/-}$TNF$^{-/-}$ mice could be utilized to simulate IgA nephropathy, and aspirin could alleviate the IgA deposits and prohibit platelet-mediated B cells non-canonical NF-κB activation in TBK1$^{-/-}$TNF$^{-/-}$ mice.

Conclusions

The inhibition of platelets dependent noncanonical NF-kB-mediated B cells activation and IgA production may contribute to the alleviation of IgA nephropathy induced by aspirin administration.

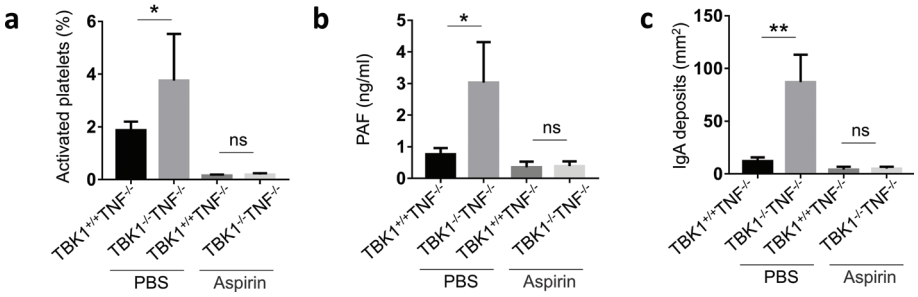

Fig. 3. Aspirin suppressed the deposition of IgA in glomeruli caused by TBK1 deficiency. The WT and TBK1$^{-/-}$TNF$^{-/-}$ mice were treated with aspirin (200 mg/kg) each week for three times. (a) Platelet activation in the plasma of the mice treated as above with PMA. (b) The protein level of PAF in the serum from aspirin-treated WT and Tbk1$^{-/-}$TNF$^{-/-}$ double knockout mice were assessed by ELISA assay. (c) Quantification of immunoglobulin deposits. The data were expressed as means ± standard error of mean (SEM). Results represent mean values of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

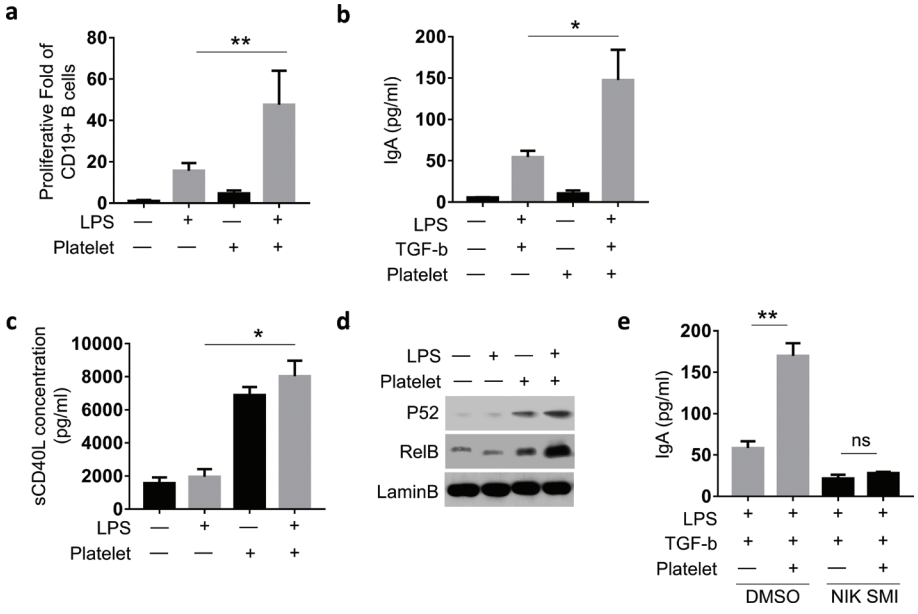

Fig. 4. Platelets promoted IgA induction in B cells *via* regulating non-canonical NF-κB. (a) Splenic B cells were stimulated for 48 h either in the absence or presence of LPS (100 ng/mL) plus 1:1 the supernatant of platelets. Cell proliferation assays of splenic T cells were evaluated by CCK8. (b) Splenic B cells were cultured for 5 days in the presence of 1 μg/mL LPS alone or in combination with 2 ng/mL TGF-β or the supernatant (1:1). IgA in cells supernatants was evaluated by ELISA. (c) The protein level of secreted CD40L in the supernatant of B cells stimulated for 48 h with of LPS (100 ng/mL) plus 1:1 the supernatant of platelets by ELISA. (d) Westernb blot analysis of non-canonical NF-κB signal in the nuclear extract of B cells treated as indicated. (e) Splenic B cells were treated as indicated plus NIK inhibitor (0.5 nM). IgA in supernatants of the cells was evaluated by ELISA. The data were expressed as means ± standard error of mean (SEM). Results represent the mean values of three independent experiments. *, $P < 0.05$; **, $P < 0.01$

Conflict of interest and funding

All the authors declare that they have no conflict of interests to declare. The study was supported by Start-up Funding for New Investigators (2020.v69.c).

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