Original Research

The regulatory T cell effector soluble fibrinogen-like protein 2 induces tubular epithelial cell apoptosis in renal transplantation

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Abstract

Acute rejection (AR) hinders renal allograft survival. Tubular epithelial cell (TEC) apoptosis contributes to premature graft loss in AR, while the mechanism remains unclear. Soluble fibrinogen-like protein 2 (sFGL2), a novel effector of regulatory T cells (Treg), induces apoptosis to mediate tissue injury. We previously found that serum sFGL2 significantly increased in renal allograft rejection patients. In this study, the role of sFGL2 in AR was further investigated both *in vivo* and *in vitro*. The serum level of sFGL2 and the percentage of $CD4^+CD25^+Foxp3^+$ Treg in the peripheral blood were measured in renal allograft recipients with AR or stable renal function ($n = 30$ per group). The human TEC was stimulated with sFGL2, tumor necrosis factor (TNF)-α, or phosphate buffered saline and investigated for apoptosis *in vitro*. Apoptosis-associated genes expression in TEC was further assessed. Approval for this study was obtained from the Ethics Committee of Fudan University. Our results showed that the serum level of sFGL2, correlated with Treg in the peripheral blood, was significantly increased in the AR patients. *In vitro*, sFGL2 remarkably induced TEC apoptosis, with a significant up-regulation of proapoptotic genes, including *CASP-3*, *CASP-8*, *CASP-9*, *CASP-10*, *TRADD*, *TNFSF10*, *FADD*, *FAS*, *FASLG*, *BAK1*, *BAD*, *BAX,* and *NF-KB1*. However, no significant changes were observed in the expression of antiapoptotic genes, including *CARD-18*, *NAIP*, *BCL2*, *IKBKB,* and *TBK1*. Therefore, sFGL2, an effector of Treg, induces TEC apoptosis. Our study suggests that sFGL2 is a potential mediator in the pathogenesis of allograft rejection and provides novel insights into the role of Treg in AR.

Keywords: Soluble fibrinogen-like protein 2, tubular epithelial cells, apoptosis, renal transplantation, regulatory T cells

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Introduction

+Renal transplantation has merged as a viable therapeutic modality for the treatment of end-stage renal disease. Acute rejection (AR) is considered as an impediment to short- and long-term survival of both renal allografts and recipients.[1] Tubular epithelial cells (TECs) compromise over 80% of renal parenchymal cells and play a critical role in maintaining normal kidney function.[2] TECs are the primary focus of allograft rejection,[3] whose injury is one of the most persistent cellular markers in the pathologic analysis of renal allograft rejection.[4] However, the classic lesion of tubular necrosis is not adequate to describe the consequences of various damages on decreased renal function. Experimental evidence supports a pathogenic role for apoptosis in TEC injury.[5] In fact, TEC apoptosis is one of the prominent features in AR[2] and contributes to graft loss,[6] while the mechanism has not been fully clarified. Therefore, a better understanding of TEC apoptosis and its mechanism in AR will promote targeting interventions and benefit allograft survival.

Apoptosis is a process of programmed cell death serving as a defense mechanism to remove unwanted and potentially dangerous cells.[7] There are two broad pathways leading to apoptosis, the extrinsic and intrinsic pathways.

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In both pathways, signaling results in the activation of a family of cysteine proteases, named caspases, that acts in a proteolytic cascade to dismantle and remove the dying cells. However, there also exist various antiapoptotic pathways, such as Bcl-2 family. A delicate balance between pro- and antiapoptotic mechanisms determines whether a cell death signal can activate the execution of the apoptotic program.[8] In addition, regulatory signal proteins, including nuclear factor-kappa-B (NF-κB), take part in the modulation of apoptosis. Therefore, apoptosis is a complex process involving multiple factors.

Soluble fibrinogen-like protein 2 (sFGL2) is a novel molecule found to induce cellular apoptosis. Identified as a member of fibrinogen-related proteins superfamily,[9] sFGL2 is mainly secreted by regulatory T cells (Treg)[10] and presents contradictory properties in tissue injuries. Recent studies show that sFGL2 maintains Treg activity and demonstrates immunosuppressive function to protect against tissue injuries.[11–14] However, it has also been reported that sFGL2 promotes cellular apoptosis, such as sinusoidal endothelial cells and hepatocytes, through binding to the inhibitory FcγRIIB receptor on the cell surface and leads to tissue injuries.[15,16] In the previous study, we found that serum sFGL2 significantly increased in renal allograft recipients with AR, which was induced by proinflammatory cytokines TNF-α and IFN-γ.[17,18] But the effects of sFGL2 on the graft remain unclear. Therefore, in this study, we examined the serum level of sFGL2 and the percentage of $CD4^+CD25^+Foxp3^+$ Treg in the peripheral blood in renal allograft recipients with AR. The effects of sFGL2 on TEC apoptosis and the expression of apoptosis-associated genes in the activated TECs were further investigated.

Materials and methods

Baseline characteristics of the patients

Sixty living-related renal transplant recipients from January 2010 to December 2012 were collected in this study. All of them received allograft biopsies, either protocol or clinically dictated due to increasing serum creatinine. Among them, 30 recipients were found AR based on the Banff 2007 classification,[19] while the others maintained stable renal function without discovery of rejection for at least 6 months. All patients were administered with triple immunesuppressants, including mycophenolate mofetil, cyclosporine A or tacrolimus, and prednisone. Patients were excluded if they had fever of undetermined origin, pneumonia, and other causes of renal graft dysfunction (e.g. infection, pyelonephritis, immunosuppressant toxicity). All living-related renal transplant recipients and this study were approved by the Ethics Committee of Fudan University (Shanghai, China). Procedures in this study were in accordance with the Helsinki Declaration of 1975, with informed consents.

sFGL2 detected by enzyme-linked immunosorbent assay (ELISA)

Blood of the patients was centrifuged at 4°C, 2500 rpm, for 25 min to obtain the serum. Serum level of sFGL2 was detected in duplicate using an ELISA kit (Biolegend, San Diego, CA, USA). The sample preparation and procedure were performed according to the manufacturer's instructions.

$CD4^+CD25^+Foxp3^+$ Treg detected by flow cytometry

Fresh heparinized peripheral blood was collected from the patients. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation, surface stained with CD4-FITC, CD25-APC, and subsequently intracellular stained with Foxp3-PE, and detected by flow cytometry using the BD fluorescent-activated cell sorter (FACS) system (BD Bioscience, San Jose, CA, USA) as previously described.[20] The sample preparation and procedure were performed according to the manufacturer's instructions. Primary data were analyzed by Flowjo software version 7.6 (Tree Star, Inc., Ashland, OR, USA).

TEC stimulation and apoptosis detection

Human renal proximal TECs (3×10^4) were bought from Sciencell (Carlsbad, CA, USA) and stimulated with 1, 10, or 100 μg/mL human sFGL2 recombinant protein (Abnova, Taipei, Taiwan), 10 ng/mL tumor necrosis factor (TNF)-α (R&D systems, Boston, MA, USA) as positive control[21] or phosphate buffered saline (PBS) as negative control for 48 h. Cell apoptosis was detected by an Annexin V-FITC Apoptosis Detection Kit (Merck, Darmstadt, German), using the BD FACS system. The sample preparation and procedure were performed according to the manufacturer's instructions. Data were analyzed by Flowjo software version 7.6. The experiment was repeated for at least 3 times.

Apoptosis-associated genes detected by real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from TECs with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (3–5 μg) was reverse transcribed into complementary DNA by Superscript II reverse transcriptase (Invitrogen) and random primer oligonucleotides (Invitrogen). Gene-specific primers for human *CASP-3*, *CASP-8*, *CASP-9*, *CASP-10*, inhibitory caspase recruitment domain family member 18 (*CARD-18*), nucleotide-binding apoptosis inhibitory protein (*NAIP*), TNF receptor-1-associated death domain (*TRADD*), *TNFSF10* (TNF superfamily member 10, also known as TNF-related apoptosis-inducing ligand, TRAIL), Fas-associated death domain (*FADD*), *FAS*, Fas ligand (*FASLG*), *BAK1*, *BAD*, *BAX*, *BCL2*, *NFKB1*, NFKB inhibitor kinase B (*IKBKB*), Tank-binding kinase 1 (*TBK1*), and *GAPDH* were designed based on sequences available through the National Center for Biotechnology Information (NCBI) database (for sequences, see Supplement Table 1). RT-qPCR was performed using the Absolute QPCR SYBR Green premix (Takara Bio Inc., Tokyo, Japan) in MasterCycler RealPlex4 system (Eppendorf, Hamburg, Germany). After a hot start (30 s at 95°C), the parameters for amplification were as follows: 5 s at 95°C, 30 s at 55°C, and 60 s at 72°C for 45 cycles.

Expression level normalized with *GAPDH* was calculated against unstimulated TECs using a $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Baseline characteristics and data are presented as mean ± standard error of the mean (SEM) and frequencies (percentages). SPSS 18.0 software (SPSS Inc., Armonk, NY, USA) was used for data analysis. The two-tailed independent Student's *t*-test after the demonstration of homogeneity of variance with F test was used to compare the continuous variables between two groups, while Pearson's χ^2-test was used for the categorical data. One-way ANOVA was used for statistics for more than two groups, and *post hoc* comparisons were then performed by Scheffe test. Spearman correlation coefficients were also used to determine the strength of relationships between parameters. $P < 0.05$ was considered as statistically significant.

Results

Characteristics of renal allograft recipients

Sixty living-related renal allograft recipients were collected in this study and divided into the AR and stable renal function groups, according to the results of renal allograft biopsies. Twenty-eight of the AR patients received biopsy due to their increasing serum creatinine, while two of them were accidentally found AR during protocol biopsy. On the other hand, 28 of patients in the stable renal function group were confirmed by protocol biopsy, whereas only two of them received biopsy because of the increasing serum creatinine but no evidence of rejection was found. Baseline characteristics analysis showed no significant differences in mean age, sex, induction therapy, cold ischemia time, immunesuppressants, or post-transplantation time between two groups. In addition, AR patients exhibited a remarkably higher level of serum creatinine at the time of biopsy, compared to those with stable renal function (Table 1).

Serum level of sFGL2 and the peripheral percentage of Treg in renal allograft recipients

Serum level of sFGL2 was significantly increased in the AR patients compared to those with stable renal function (Figure 1(a)), which was similar to our previous study.[17] In addition, the peripheral percentage of $CD4^+CD25^+$ $Foxp3^+$ Treg was also significantly increased in the AR patients (Figure 1(b)). Furthermore, we found that serum sFGL2 level was positively correlated with peripheral Treg proportion (Spearman correlation coefficient 0.715, $P < 0.001$).

TECs stimulated with sFGL2 in vitro

We further investigated the effects of sFGL2 on TECs by stimulating TECs with sFGL2, TNF-α, or PBS *in vitro* and

Table 1 Baseline characteristics

	AR ($n = 30$)	STA ($n = 30$)	*P* value
Age (year)[a]	25.60 ± 1.54	26.37 ± 1.12	>0.05
Sex[b]			
Male	17 (57%)	16 (53%)	>0.05
Female	13 (43%)	14 (47%)	>0.05
Induction therapy[b]			
CD25 mAb	28 (93%)	27 (90%)	>0.05
ATG	2 (7%)	3 (10%)	>0.05
Cold ischemia time (hour)[a]	6.73 ± 0.19	6.87 ± 0.13	>0.05
Immunosuppressants[b]			
MMF + CsA + Pre	15 (50%)	16 (53%)	>0.05
MMF + FK506 + Pre	15 (50%)	14 (47%)	>0.05
Post-transplantation time (year)[a]	2.39 ± 0.19	2.28 ± 0.18	>0.05
Scr at biopsy (μmol/L)[a]	175.83 ± 6.10	100.47 ± 2.75	<0.001
Biopsy[b]			
Protocol	2 (6.7%)	28 (93.3%)	<0.001
Clinical dictated[c]	28 (93.3%)	2 (6.7%)	<0.001
Banff category			
IA	5 (16.7%)	0	–
IB	10 (33.3%)	0	–
IIA	8 (26.7%)	0	–
IIB	5 (16.7%)	0	–
IIIA	2 (6.7%)	0	–

[a]Mean ± SEM.
[b]Frequency (percentage).
[c]Clinical-dictated biopsy was taken due to the increase of serum creatinine.
AR: acute rejection; STA: stable renal function; mAb: monoclonal antibody; ATG: antithymocyte globulin; MMF: mycophenolate mofetil; CsA: cyclosporine A; FK506: tacrolimus; Pre: prednisone; Scr: serum creatinine.

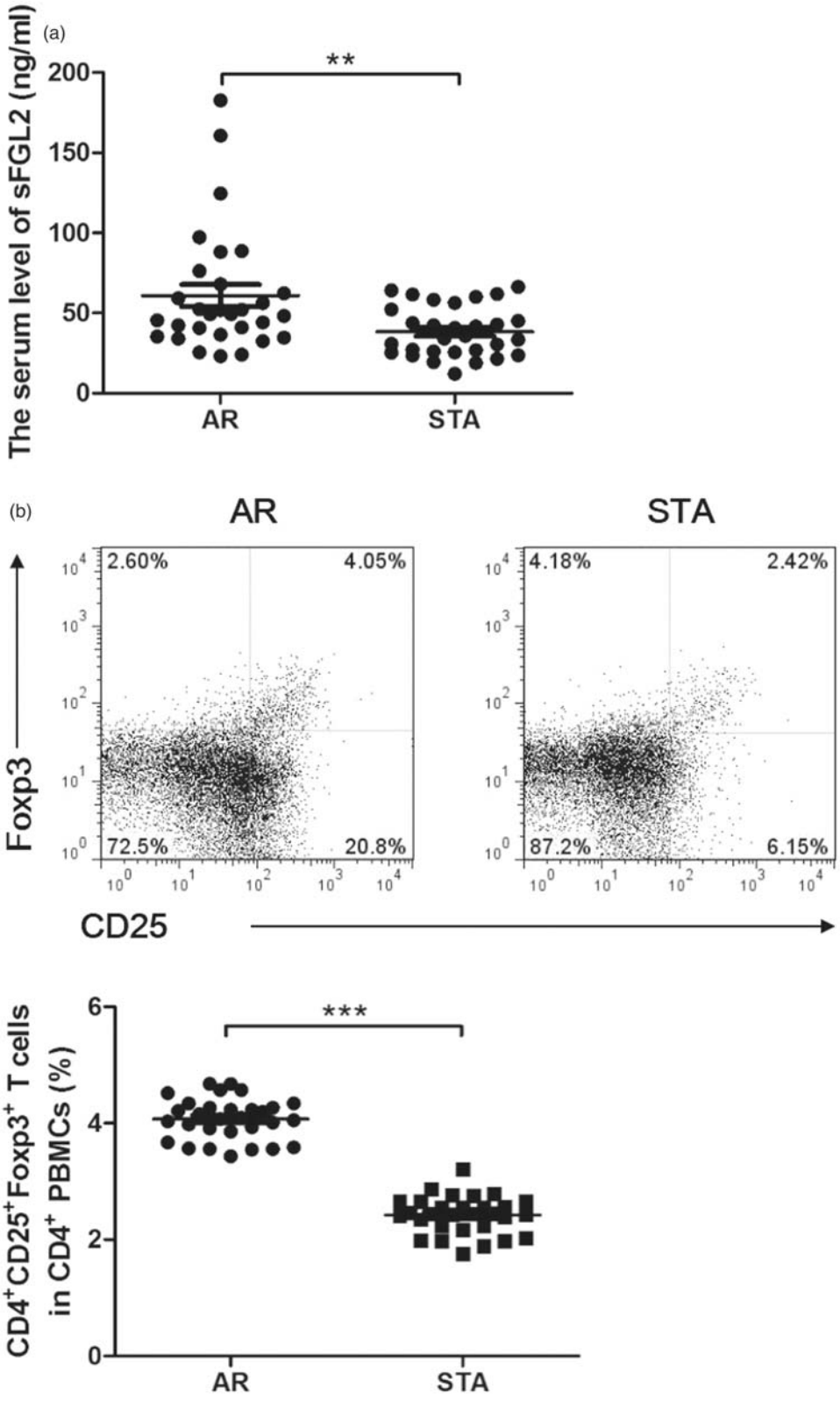

Figure 1 sFGL2 and Treg in the peripheral blood of renal allograft recipients. The serum level of sFGL2 (60.94 ± 6.93 versus 38.44 ± 2.86 ng/ml) (a) and the percentage of $CD4^+CD25^+Foxp3^+$ Treg in $CD4^+$ PBMCs (4.07 ± 0.07 versus $2.42 \pm 0.06\%$) (b) were significantly increased in renal allograft recipients with acute rejection (AR), compared to those with stable renal function (STA). Data are expressed as mean $\pm$ SEM ($n = 30$). **: $P < 0.01$; ***: $P < 0.001$

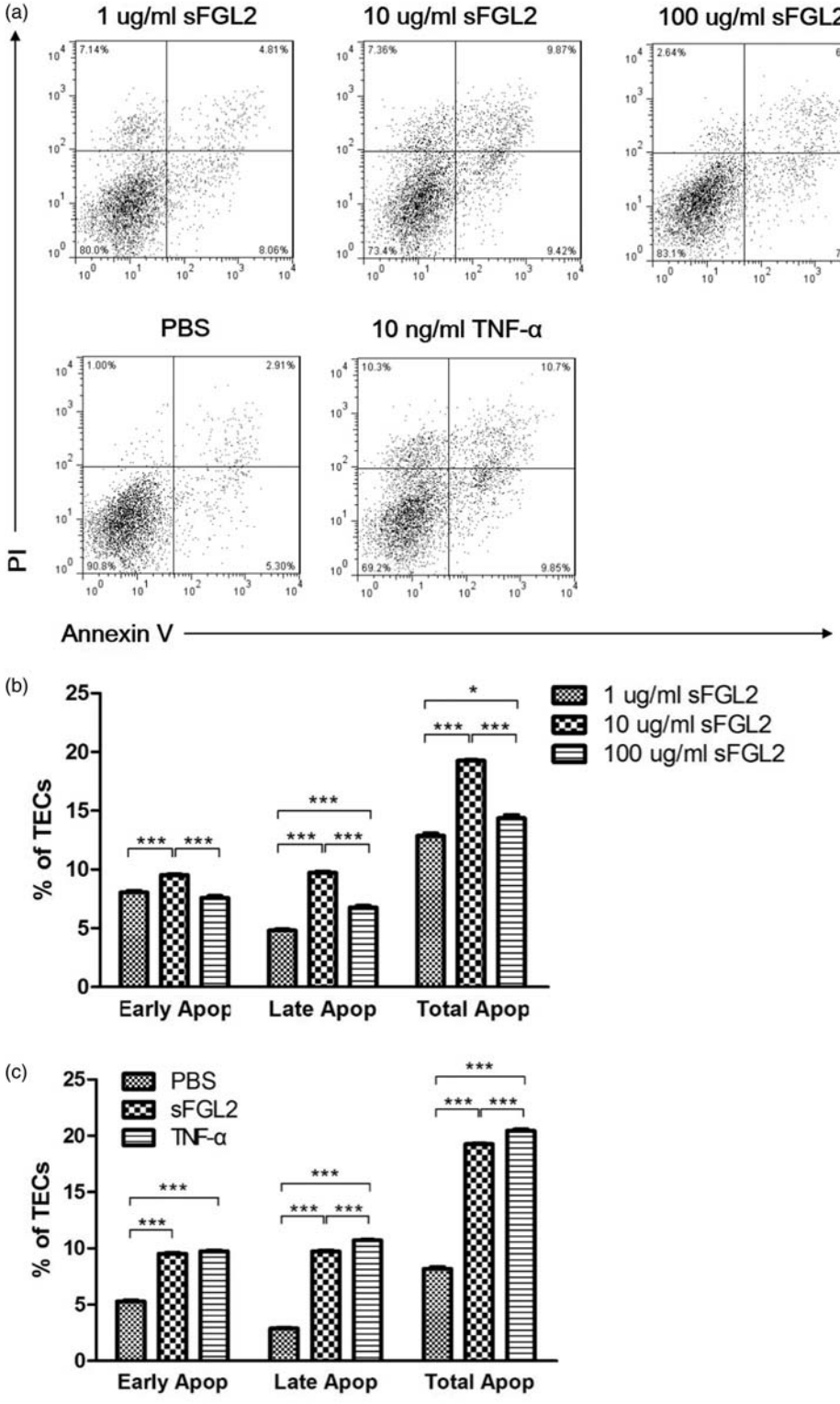

Figure 2 sFGL2-induced TEC apoptosis *in vitro*. TECs were stimulated with 1, 10, or 100 μg/mL sFGL2, 10 ng/mL TNF-α as positive control or PBS as negative control for 48 h, and detected for apoptosis with Annexin V/PI staining by flow cytometry (a). The proportion of apoptotic cells in TEC was significantly higher with sFGL2 stimulation at 10 μg/mL than 1 and 100 μg/mL (19.25 ± 0.06%, 12.86 ± 0.23%, and 14.37 ± 0.27%), including both early (9.54 ± 0.06%, 8.05 ± 0.12%, and 7.60 ± 0.14%) and late (9.72 ± 0.10%, 4.81 ± 0.10%, and 6.77 ± 0.14%) apoptotic cells (b). Further, the proportion of apoptotic cells in TEC was significantly higher with 10 μg/mL sFGL2 stimulation than PBS (8.18 ± 0.16%), including both early (5.29 ± 0.10%) and late (2.89 ± 0.06%) apoptotic cells. Moreover, TNF-α significantly increased the proportion of apoptotic cells (20.45 ± 0.12%), including early (9.74 ± 0.09%) and late apoptotic cells (10.71 ± 0.04%), in comparison with PBS. In addition, the proportion of total and late apoptotic cells was significantly higher in the TNF-α group than the sFGL2 group, while there was no significant difference in the proportion of early apoptotic cells between these two groups (c). Data are expressed as mean ± SEM. *: $P < 0.05$; ***: $P < 0.001$

detected for apoptosis with Annexin V/propidum iodide (PI) staining by flow cytometry (Figure 2(a)). Annexin V^+/PI^- and Annexin V^+/PI^+ phenotypes represent early and late apoptotic cells, respectively.[22] We found that TECs presented the most percentage of apoptosis when stimulated with 10 μg/mL sFGL2, compared to other doses (Figure 2(b)). Thus, we further compared 10 μg/mL sFGL2 with 10 ng/mL TNF-α and PBS in the potency of inducing TEC apoptosis. It showed that the proportion of apoptotic cells in TECs was significantly higher with sFGL2 or TNF-α stimulation than PBS, including both early and late apoptotic cells. In addition, the proportion of total and late apoptotic cells was significantly higher in the TNF-α group than that in the sFGL2 group, while there was no significant difference in the proportion of early apoptotic cells between these two groups (Figure 2(c)). These findings demonstrated the capacity of sFGL2 in promoting TEC apoptosis.

Expression of apoptosis-associated genes in the activated TECs

The expression of apoptosis-associated genes in the activated TECs was further detected by RT-qPCR (Supplement Table 2). In comparison with PBS, sFGL2 promoted a significant up-regulation of proapoptotic genes, including *CASP-3, CASP-8, CASP-9, CASP-10, TRADD, TNFSF10, FADD, FAS, FASLG, BAK1, BAD, BAX,* and *NF-KB1*. However, no significant changes were observed between the sFGL2 group and the PBS group in the expression of antiapoptotic genes, including *CARD-18*, *NAIP*, *BCL2*, *IKBKB,* and *TBK1*. Compared to TNF-α, sFGL2 induced a significantly higher expression of proapoptotic genes, including *TRADD*, *TNFSF10*, *FADD*, *FAS*, *BAK1*, *BAX,* and *NFKB1*. The expression of antiapoptotic *BCL2* was also significantly increased in the sFGL2 group. No significant changes were observed between the sFGL2 group and the TNF-α group in the expression of other genes. Furthermore, TNF-α stimulation led to a significantly lower expression of *TRADD*, *TNFSF10*, *BAK1,* and *BAX*, without any significant changes in other proapoptotic genes and antiapoptotic genes (Figure 3). These findings indicated that sFGL2 induced TEC apoptosis through up-regulation of proapoptotic genes, without down-regulation of antiapoptotic genes.

Discussion

TEC apoptosis participates in the pathogenesis of AR after kidney transplantation. This study demonstrated that sFGL2, a novel trigger of apoptosis, significantly increased in the AR patients in accordance with the peripheral proportion of Treg. Further *in vitro* investigations showed that

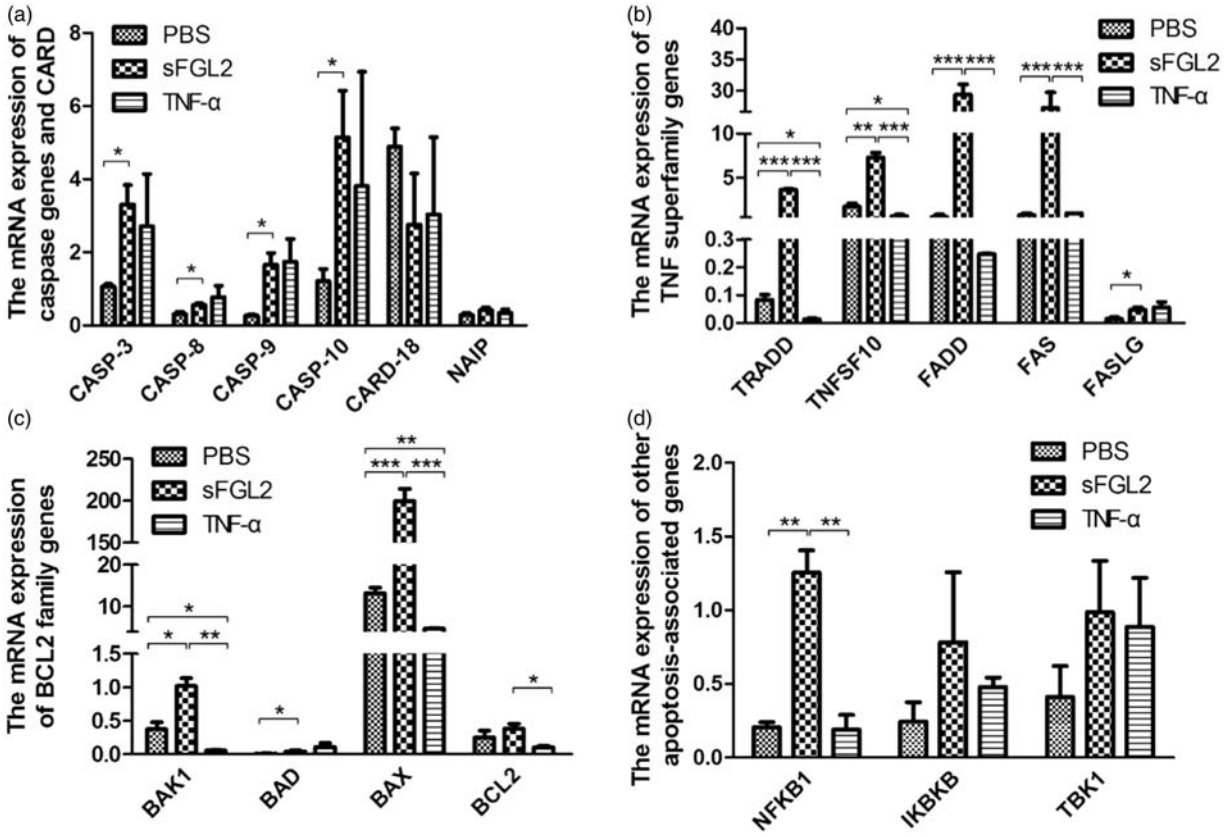

Figure 3 Apoptosis-associated genes expression in TECs. Compared to the negative control, sFGL2 promoted a significant up-regulation of proapoptotic genes, including *CASP-3, CASP-8, CASP-9, CASP-10, TRADD, TNFSF10, FADD, FAS, FASLG, BAK1, BAD, BAX,* and *NF-KB1*, while no significant changes were observed in the expression of antiapoptotic genes, including *CARD-18*, *NAIP*, *BCL2*, *IKBKB,* and *TBK1*. Compared to TNF-α, sFGL2 induced a significant higher expression of proapoptotic genes, including *TRADD*, *TNFSF10*, *FADD*, *FAS*, *BAK1*, *BAX,* and *NFKB1*, as well as antiapoptotic *BCL2*. Furthermore, TNF-α stimulation led to a significantly lower expression of *TRADD*, *TNFSF10*, *BAK1,* and *BAX*, compared to PBS. Data are expressed as mean ± SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

sFGL2 induced TEC apoptosis through up-regulation of proapoptotic genes without down-regulation of antiapoptotic genes, in the expression of mRNA levels of caspases, TNF superfamily, Bcl-2 family, and NF-κB-related proteins. Our findings suggest that sFGL2 plays an important role in renal allograft rejection.

It is commonly recognized that Treg contributes to the immune tolerance of allografts.[23,24] Treg increased in AR to ameliorate inflammation and rejection injury through its immunoregulatory function.[25,26] sFGL2 is a novel effector of Treg and has been proved to associate with Treg in several diseases. In fulminant viral hepatitis in mice, the elevated peripheral sFGL2 level correlates with increasing percentage of Treg.[27] In tolerant heart and liver allografts in rats, the FGL2 mRNA increased in accordance with Foxp3, the classic marker of Treg.[28] Furthermore, serum and tissue FGL2 has been proved to be a surrogate for Treg activity in patients with chronic viral hepatitis C.[29] In our study, we first confirmed the strong correlation of sFGL2 and Treg in AR by revealing their synchronical increase and significant correlation coefficient in the peripheral blood of renal allograft recipients. Therefore, sFGL2 may act as a mediator of Treg activity, and its role in AR indicates the effects of Treg on the graft.

Soluble FGL2 has been proven to induce apoptosis. As a multistage programmed cell death, apoptosis features with nuclear and cytoplasmic condensation, followed by plasma membrane blebbing and release of apoptotic bodies, which are rapidly identified by neighboring cells or professional phagocytes and disposed generally without induction of inflammation.[30] The apoptosis process includes an early stage that involves cellular membrane phosphatidylserine (PS) exposure and a late stage that pertains to the degradation of genomic DNA,[31] stained typically by Annexin V and PI, respectively.[32,33] In this study, sFGL2 induced TEC apoptosis in both early and late stages. TNF-α promoted stronger total, especially late, apoptosis of TECs compared to sFGL2. However, sFGL2 exhibited a similar potent property in triggering early apoptosis of TEC as TNF-α. It is known that early exposure of membrane PS is responsible for the phagocytic disposal of injured cells.[31] Therefore, our results suggest that sFGL2 might promote a quick phagocytosis of apoptotic cells and lead to a limited inflammation.

In this study, sFGL2 induced TEC apoptosis through up-regulation of proapoptotic genes without down-regulation of antiapoptotic genes, in the expression of mRNA levels of caspases, TNF superfamily, Bcl-2 family, and NF-κB-related proteins. It is known that apoptosis results from the interactions of proteins from a complicated network, involving two main molecular pathways.[34] The extrinsic apoptosis indicates a form of death induced by extracellular signals that result in the binding of ligands to specific trans-membrane receptors, collectively known as death receptors belong to the TNF superfamily, and recruiting the downstream adaptor proteins, such as TRADD. Fas/Fas ligand and its adaptor molecule FADD are listed as one of the most well-known signalings in the pathway. The intrinsic apoptosis, on the other hand, is activated in response to a number of stressing conditions, and leads to the mitochondria dysfunction which releases the cytochrome C. The caspases play a crucial role in the execution or final phase of cell death by cleaving and inactivating various structural and functional intracellular proteins that are essential for cell survival and proliferation.[35] Caspases-8 and -10 function in the extrinsic pathway and caspases-9 in the intrinsic pathway as the initiator caspases, while caspase 3 acts as the executioner caspase in the final degradation part. The activity of caspases is suppressed by a series of inhibitors, including NAIP and CARD-18,[36,37] while their activation is under the tight control of the Bcl-2 family proteins.[7] Bcl-2 family mainly acts by regulating the release of caspases activators from mitochondria, involving two groups of proteins with opposing effects. The proapoptotic group mediates apoptosis by disrupting membrane integrity, including Bak, Bad, and Bax, while the antiapoptotic group, such as Bcl-2, prevents apoptosis by interfering with proapoptotic member aggregation.[38] Moreover, NF-κB is another pathway responsible for apoptosis activation, and its function is inhibited by regulators such as IKBKB and TBK1.[39] Through detection of apoptosis-associated genes in the activated TECs, this study demonstrated that sFGL2 promoted not only extrinsic, but also intrinsic apoptotic pathways. Both initiators and executioners in the caspase cascade, the central point of apoptosis, were up-regulated in TECs with sFGL2 stimulation. In addition, the regulatory signals, such as Bcl-2 and NF-κB family, were modulated by sFGL2 toward the proapoptosis profile as well. Therefore, sFGL2 induces TEC apoptosis through a comprehensive proapoptotic facilitation.

Recent studies show that TEC apoptosis occurs in two distinct phases. The first phase occurs early on 12–48 h after the injury, on which TEC apoptosis contributes to tubular cell loss and the tubular dysfunction associated with kidney injury. While the second phase occurs many days later, during the recovery process, with TEC apoptosis postulated to contribute to the remodeling of injured tubules and to facilitate their return to a normal structural and functional state.[40] Moreover, apoptotic cells and bodies are rapidly phagocytized, thereby protecting surrounding tissues from injury.[41] Therefore, the consequence of TEC apoptosis in AR depends on its time phase. Since sFGL2 is mainly secreted by Treg, its induction of TEC apoptosis on the early stage of injury might reveal a destructive effect of Treg, which provides a novel insight for the role of Treg during AR. On the other hand, if sFGL2 promotes TEC apoptosis on the late recovery stage, this impact might be considered as another renoprotective mechanism of Treg, apart from their immunosuppressive property. Therefore, the distinct effects of sFGL2 in different time phases during AR need further investigations. Furthermore, as an immunoregulatory effector of Treg, sFGL2 can inhibit dendritic cell maturation and induce B cell apoptosis *in vitro*.[42] Since apoptosis of different type of cells could lead to different clinical outcomes, the final impact of sFGL2 on the renal allograft depends on its apoptosis-inducing potency inclination. Therefore, local expression of sFGL2 in the renal allograft and its effect on TEC *in vivo* are yet to be discovered. Further interventional studies are needed as well.

In conclusion, serum level of sFGL2 was significantly increased in the AR patients in correlation with the

peripheral proportion of Treg. *In vitro*, sFGL2 remarkably promoted TEC apoptosis with a significant up-regulation of proapoptotic genes, while no significant changes were observed in the expression of antiapoptotic genes. Our findings shed light on the mechanism of sFGL2-induced TEC apoptosis, suggesting sFGL2 a potential mediator in the pathogenesis of allograft rejection and provide novel insights into the role of Treg in renal transplantation.

Author contributions: ZZ and CY designed the study and wrote the manuscript. LW performed experiments and analyzed the data; LL, TZ, and LH collected clinical data and patients' blood samples; RR, MX, and TZ participated in research design and funding support.

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