International Immunopharmacology 71 (2019) 32–39

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Thalidomide ameliorate graft chronic rejection in an allogenic kidney transplant model

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ARTICLE INFO

Keywords:
Thalidomide
Chronic rejection
T cell mediated rejection
Kidney transplantation
Treg
Th17 cell

ABSTRACT

Chronic T cell mediated rejection (TCMR), which is characterized by infiltration of the interstitium by T cells and macrophages, still remains a major barrier to the long-term survival of kidney transplantation. Our recent report indicated that thalidomide can attenuate graft arteriosclerosis in an aortic transplant model. In this study, we investigated the effect of thalidomide on chronic TCMR in a rat model of kidney transplantation.

Fischer or Lewis kidney allografts were transplanted into Lewis recipient rats. After kidney transplantation, recipient rats were divided into 3 groups: the isograft (Iso) group, allograft (Allo) group, and thalidomide (Tha) group. Rats were sacrificed at 8 weeks after kidney transplantation, and blood and kidney samples were collected. Serum concentrations of creatinine (SCr),interleukin (IL)-2, IL-6, IL-17, and TNF-α in recipients were determined, and flow cytometry was used to detect the percentages of $CD4^+CD25^+$, $CD4^+$ $Foxp3^+$and $CD4^+Th17^+$ cell subsets in the peripheral blood. Grafts were procured for histopathological examination, and the expressions of α-SMA, transforming growth-β1 (TGF-β1), and VEGF in kidney grafts were investigated using Western blot. Thalidomide treatment significantly ameliorated chronic rejection, reduced renal allograft tissue damage, and decreased serum creatinine levels. Attenuation of chronic TCMR was due to the prohibited production of inflammatory cytokines, altered distribution of the $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T (Treg) and $CD4^+$ $Th17^+$ cells in the peripheral blood, and decreased expression of TGF-β1, α-SMA, and VEGF in the kidney graft. These results demonstrated that thalidomide could effectively ameliorate chronic TCMR in a rat kidney transplant model.

1. Introduction

Kidney transplantation (KT) is an effective therapy for patients with end-stage renal disease (ESRD). KT is generally considered the best treatment for patients with ESRD due to the improved quality of life and cost-effectiveness [1]. Although the use of cyclosporine and tacrolimus significantly reduces the incidence of acute graft rejection and improves the short-term prognosis after renal transplantation in most centers [2], long-term grafts and patient survival are not improved significantly [3]. One of the main causes of graft loss is chronic rejection, which is defined as the histopathological features of chronic interstitial fibrosis, tubular atrophy, graft arteriosclerosis, and glomerular sclerosis [4].

Currently, there is no available effective therapy for chronic rejection. Therefore, improved immunosuppressive therapies and the development of novel immunosuppressive drugs are critical to reducing the risk of chronic graft rejection.

Thalidomide (α-(*N*-phthalimido) glutarimide) first appeared on the market in 1954 for the treatment of morning sickness in pregnant women with anti-emetic properties [5,6]. However, it was withdrawn from the market in 1961 because it caused birth defects in approximately 10,000 children [7]. After it had already been taken off the market, Sheskin found an anti-inflammatory and immunomodulatory trait in erythema nodosum leprosum (ENL), and an inflammatory complication of leprosy [8]. Additional studies and clinical investigations further demonstrated that thalidomide inhibited fibroblast growth factor (bFGF)-induced angiogenesis and neovascularization [6]. Moreover, thalidomide has been used to treat multiple myeloma, liver cancer, glioma, breast cancer, and renal cell carcinoma [9]. A series of studies have confirmed the immunosuppressive and anti-inflammatory effects of thalidomide on acute and chronic graft-versus-host disease (GVHD) in bone marrow transplantation [10–12]. Similarly, the recent study showed that thalidomide could prolong the survival of

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https://doi.org/10.1016/j.intimp.2018.12.035
Received 23 July 2018; Received in revised form 13 December 2018; Accepted 14 December 2018
Available online 13 March 2019

heterotopic heart allografts in animals [13]. In our previous research, thalidomide could significantly attenuate transplant arteriosclerosis in a rat aortic transplantation model [14]. Miller also confirmed that thalidomide treatment prevents cardiac allograft vasculopathy after aortic transplantation in rats [15].

Although the protective role of thalidomide has been suggested in chronic graft rejection, a clear mechanism by which thalidomide functions, especially in kidney transplantation, has not been elucidated to date. In this study, we hypothesized that thalidomide also attenuates chronic T cell mediated rejection (TCMR) in the kidney allograft. To test this, we examined the effect of thalidomide on chronic rejection in a rat kidney transplantation model and suggested the possible molecular mechanisms that may underlie these effects.

2. Methods

2.1. Experimental animals

Inbred male Fischer (F344, $RT1^{1v1}$) and Lewis (LEW $RT1^{1}$) rats were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). Rats were fed a standard diet and water ad libitum. All animal protocols were approved by the Wenzhou Medical University Animal Policy and Welfare Committee.

2.2. Rat model of renal transplantation

Kidney transplantation was performed in rats at 8–10 weeks, 200–250 g body weight, using a modified technique as previously described [16].

Briefly, the left kidney of Fischer or Lewis rat was removed as donors and perfused with chilled heparin sodium chloride solution (4 °C). Then, the Lewis recipient was treated with left native nephrectomy. An end-to-end anastomosis was performed, and the donor ureter was anastomosed to the dome of the bladder. After transplant procedures, all the recipients were given 5 mg/kg/d of cyclosporine for 10 d. After transplantation, the animals were randomly divided into 3 groups: the isograft control group (Lewis to Lewis, n = 6), the allogeneic group containing rats transplanted with allografts (Fischer to Lewis, n = 6), and the thalidomide treatment group containing rats transplanted with allografts (Fischer to Lewis, n = 6). In the isograft control group and allogeneic group, saline was administered to the recipient animals at a dosage of 1 ml daily. In the thalidomide group, recipient animals were treated with thalidomide (Selleck Chemicals, Houston, TX, USA) at a dosage of 100 mg/kg/d.

At the end of the study, 8 weeks after transplantation, the rats were humanely sacrificed, and the kidney grafts and serum samples were harvested for analyses.

2.3. The function of the transplanted kidney

Blood samples from each recipient were collected to measure the level of creatinine (SCr) using an AU5800 automatic biochemistry analyzer (Beckman Counter., Brea, CA, USA).

2.4. Flow cytometry

Lymph cell suspensions were prepared from the collected blood specimens by Ficoll density gradient centrifugation, and the concentration of lymphocytes in each sample was adjusted to 5×10^6 cells/ml. Then, the FITC-labeled anti-CD4 and PE-labeled anti-interleukin (IL) - 17 antibodies were added. Cell membranes were permeabilized to allow detection, and the ratio of $Th17^+$ cells to $CD4^+$ cells was determined. The concentration of lymphocytes was adjusted to 5×10^8 cells/ml, and then FITC-labeled anti-CD4 and PE-labeled anti-CD25 antibodies were added. After surface staining, cells were permeabilized using the Foxp3/Transcription Factor staining buffer set (biosciences, San Diego, CA) according to the manufacturer's instruction. Permeabilized cells were incubated with PE-labeled anti-Foxp3 antibody. The cells were analyzed with FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson, Rutherford, NJ). All antibodies were purchased from eBiosciences (San Diego, CA).

2.5. Histologic analysis of renal chronic rejection

Kidney grafts were fixed in buffered formalin and embedded in paraffin. Then, they were cut into 3–4 μm sections, and stained with hematoxylin-eosin and Masson's Trichrome. We graded chronic rejection according to the Banff 97 grading system. Glomerular, tubulitis, interstitial inflammation and vascular lesions (arteriolar hyalinosis and intimal arteritis) suggesting chronic rejection were semiquantitatively evaluated from 0 to 3: 0 absence of lesion, 1 slight, 2 moderate, 3 severe lesions. The overall degree of chronic rejection was calculated by adding the scores. All examinations were performed blindly by two pathologists.

The Masson's trichrome staining was used to analyze the degree of tubulointerstitial collagen deposition. Twenty cortical interstitial fields were randomly selected at 400× magnification, and the density of trichrome-positive signals was assessed using a bioimage analysis system (Bio-Profile).

2.6. Immunofluorescence

Kidney graft tissues were embedded in optimal cutting temperature (OCT) compound and rapidly frozen in liquid nitrogen prior to storage at −80 °C. Tissue sections were cut into 4 μm and mounted. Sections were soaked in serum-free protein block (Dako, Carpinteria, CA) for 1 h at room temperature. Kidney sections were then incubated with rabbit anti-TGF-β1 antibody (1:100 dilution, Abcam, Cambridge, MA), or rabbit anti-α-SMA antibody (1:200 dilution, Abcam, Cambridge, MA), followed by Alexa-488 conjugated goat anti-rabbit antibody (1:100 dilution, A11008; Invitrogen, Carlsbad, CA) for 1 h. Fluorescence was detected using a fluorescence microscope (Nikon Instruments, Melville, NY). Five tangent glomeruli were randomly selected from each specimen in each group at 400× magnification. The mean fluorescence intensity (MFI) of these glomeruli was calculated to show the relative quantity of TGF-β1 and α-SMA protein using Image-pro plus 5.0 software (Media Cybernetics, USA).

2.7. Western blot

The expression of TGF-β1, α-SMA, and VEGF in the kidney grafts were detected using Western blotting assay. The harvested kidney graft tissues were completely homogenized and lysed in RIPA buffer, and the total proteins were electrophoresed at 50 μg using a BCA kit (PIERCE). Then, isolated proteins were electrophoresed in a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Hybond C Extra, Amersham Biosciences, Little Chalfont, USA). The membranes were incubated in a blocking buffer A (PBS, 0.1% Tween-20 and 5% nonfat milk) and incubated with primary rabbit anti-rat TGF-β1, α-SMA, VEGF, and β-actin antibodies (Abcam Biotechnology, Cambridge, MA) overnight at 4 °C. Then, the membranes were incubated with a secondary peroxidase-conjugated horseradish antibody for 2 h at room temperature. Lastly, the signal was detected with an enhanced chemiluminescence kit (Westang, Shanghai, China) in accordance with the manufacturer's instruction. The intensities of the protein bands were quantified using a bioimage analysis system (Bio-Rad, USA).

2.8. Cytokine analysis

Blood samples from each recipient were used to determine the concentration of IL-2, IL-6, IL-17, and TNF-α in serum using an enzyme-

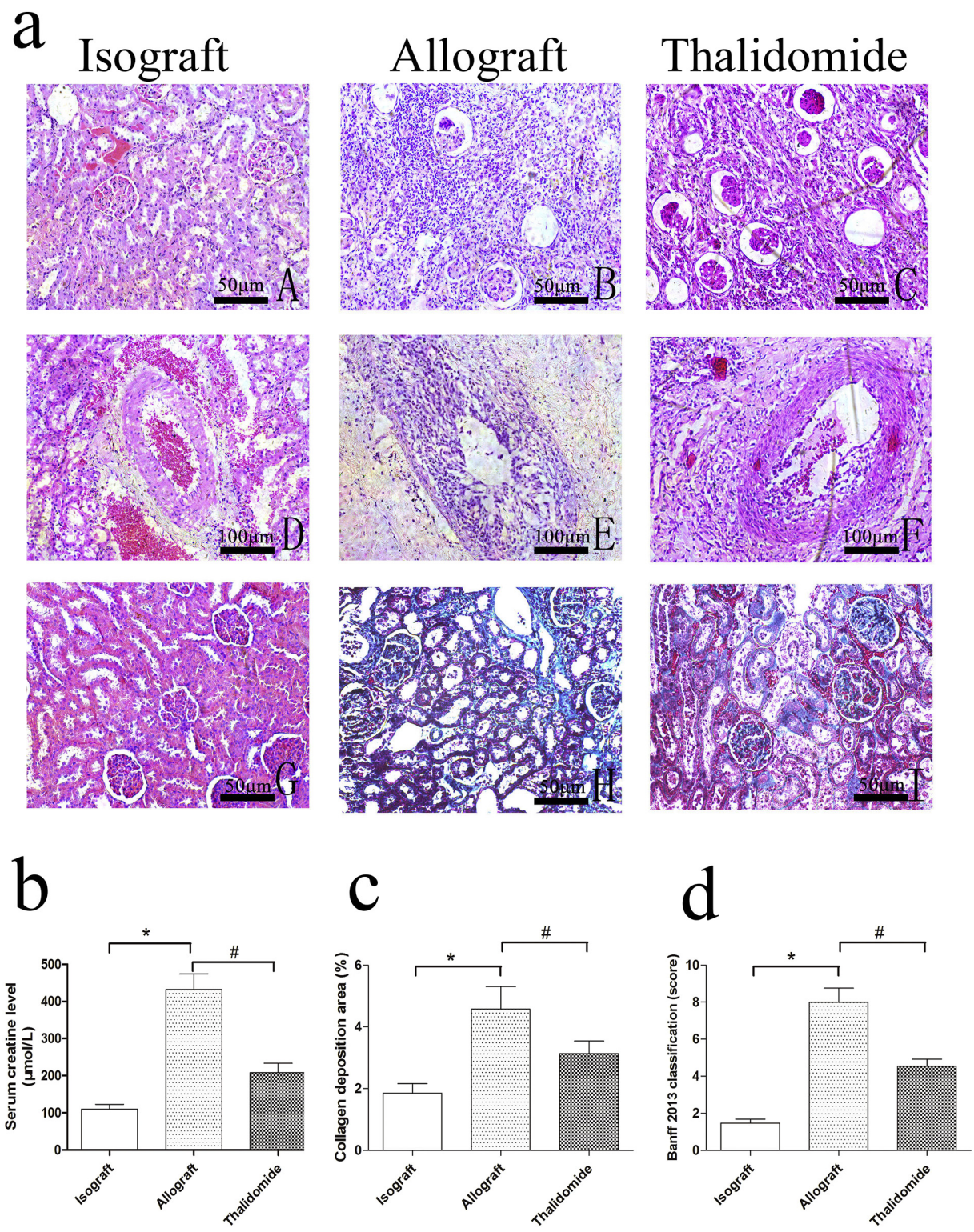

Fig. 1. (a) Thalidomide attenuated the histological changes in the transplanted kidney graft. Representative hematoxylin-eosin staining micrographs of (A, D) isograft group, (B, E) allograft group, and (C, F) thalidomide group. Representative Masson staining micrographs of the (G) isograft group, (H) allograft group, and (I) thalidomide group. (A, B, C, G, H and I) Original magnification is 200×, and scale bars represent 50 μm. (D, E, and F) Original magnification is 400×, and scale bars represent 100 μm. (b) Thalidomide improved renal function after kidney transplantation. (c) Thalidomide suppressed the tubulointerstitial collagen deposition after kidney transplantation. (d) Thalidomide decreased the Banff scores after kidney transplantation. $^{*}P < 0.05$ in comparison with the isograft group. $^{\#}P < 0.05$ in comparison with the allograft group. $N = 6$ for each group. The bar represents mean ± SEM.

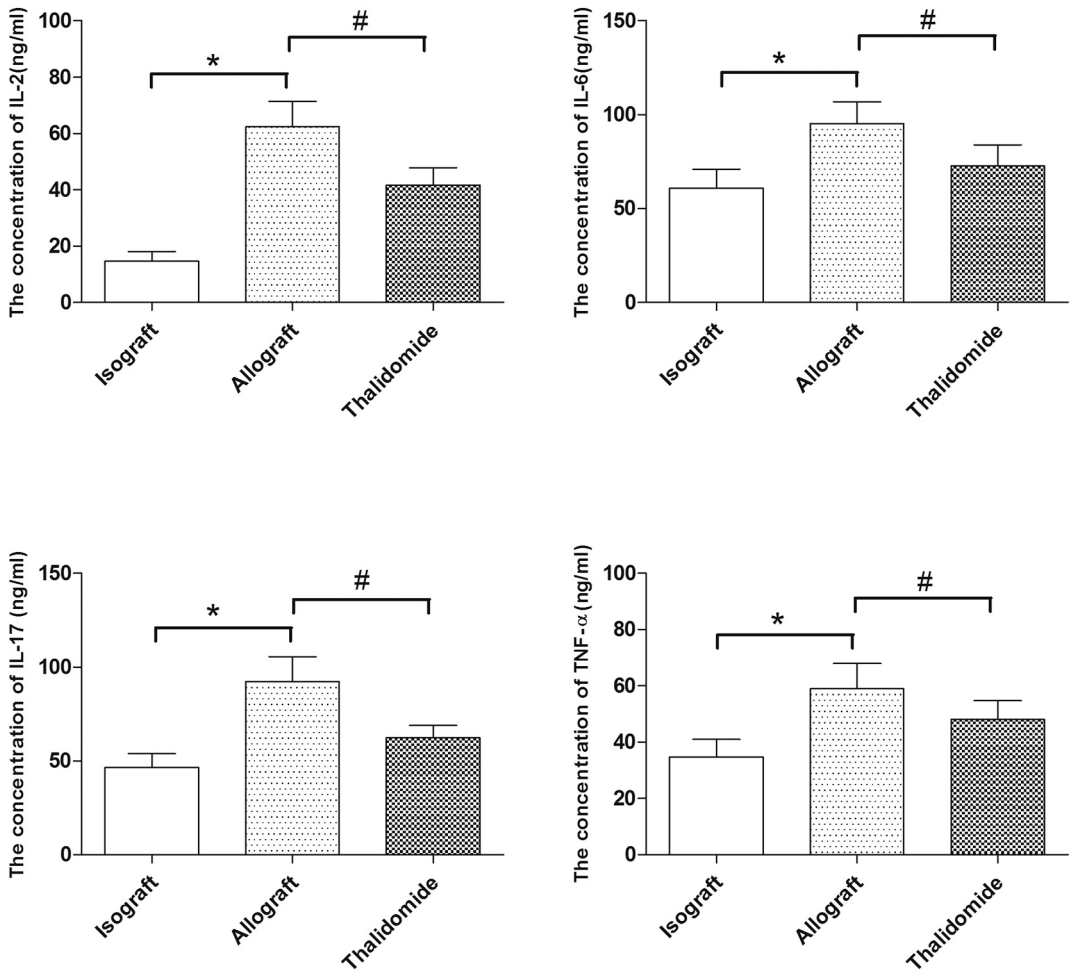

Fig. 2. Thalidomide significantly reduced the concentration of (a) IL-2, (b) IL-6, (c) IL-17, (d) TNF-α after kidney transplantation. $^{*}P < 0.05$ in comparison with the isograft group. $^{\#}P < 0.05$ in comparison with the allograft group. $N = 6$ for each group. The bar represents mean ± SEM.

linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Westang, Shanghai, China).

2.9. Statistical analysis

The results were assessed using a one-way ANOVA for comparisons between groups. Differences were assessed using the Bonferroni post-test, with $P < 0.05$ considered indicative of significant differences. Data are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Thalidomide improved renal function after kidney transplantation

At 8 weeks after transplantation, rats subjected to kidney transplantation in the allogeneic group showed a significant increase in SCr levels compared with rats in isograft control group ($P < 0.05$). The negative effects on renal function in response to kidney transplantation were significantly reduced in the thalidomide group compared with the allogeneic group, as measured by SCr levels ($P < 0.05$) (Fig. 1b).

3.2. Thalidomide attenuated the histological changes in the kidney graft after kidney transplantation

Histological examination showed chronic rejection changes, characterized by intimal proliferation of graft arteries, glomerulosclerosis, interstitial fibrosis, interstitial infiltration of lymphocytes or mononuclear cells, and tubular atrophy, in the allogeneic group after 8 weeks post transplantation (Fig. 1a, B and H), while the isograft control group did not show these pathological changes (Fig. 1a, A and G). However, the treatment with thalidomide significantly ameliorated pathological markers of chronic rejection (Fig. 1a, C and I), particularly intimal proliferation of graft arteries (Fig. 1a, D, E, and F), suggesting that thalidomide could protect allografts after kidney transplantation.

The Masson's trichrome stain of representative kidney sections also demonstrated increased collagen deposition within the tubulointerstitium in the allogeneic group after 8 weeks post transplantation (Fig. 1a, H). However, the daily treatment with thalidomide significantly suppressed the tubulointerstitial collagen deposition ($P < 0.05$) (Fig. 1a, I; b). No gross alterations were observed in the isograft control group (Fig. 1a, G; b).

Next, the pathological changes were evaluated by Banff score. The Histological analysis showed a higher Banff score in the allergenic group compared to the thalidomide group ($P < 0.05$) (Fig. 1d).

3.3. Thalidomide ameliorated systemic inflammatory environments after kidney transplantation

The serum levels of proinflammatory cytokines (IL-2, IL-6, and TNF-α) and inflammatory cytokine (IL-17) in the allogeneic group increased compared with the isograft control group ($P < 0.05$) (Fig. 2). The concentration of all proinflammatory cytokines and IL-17 was reduced in the thalidomide group compared with that in the allogeneic group ($P < 0.05$) (Fig. 2).

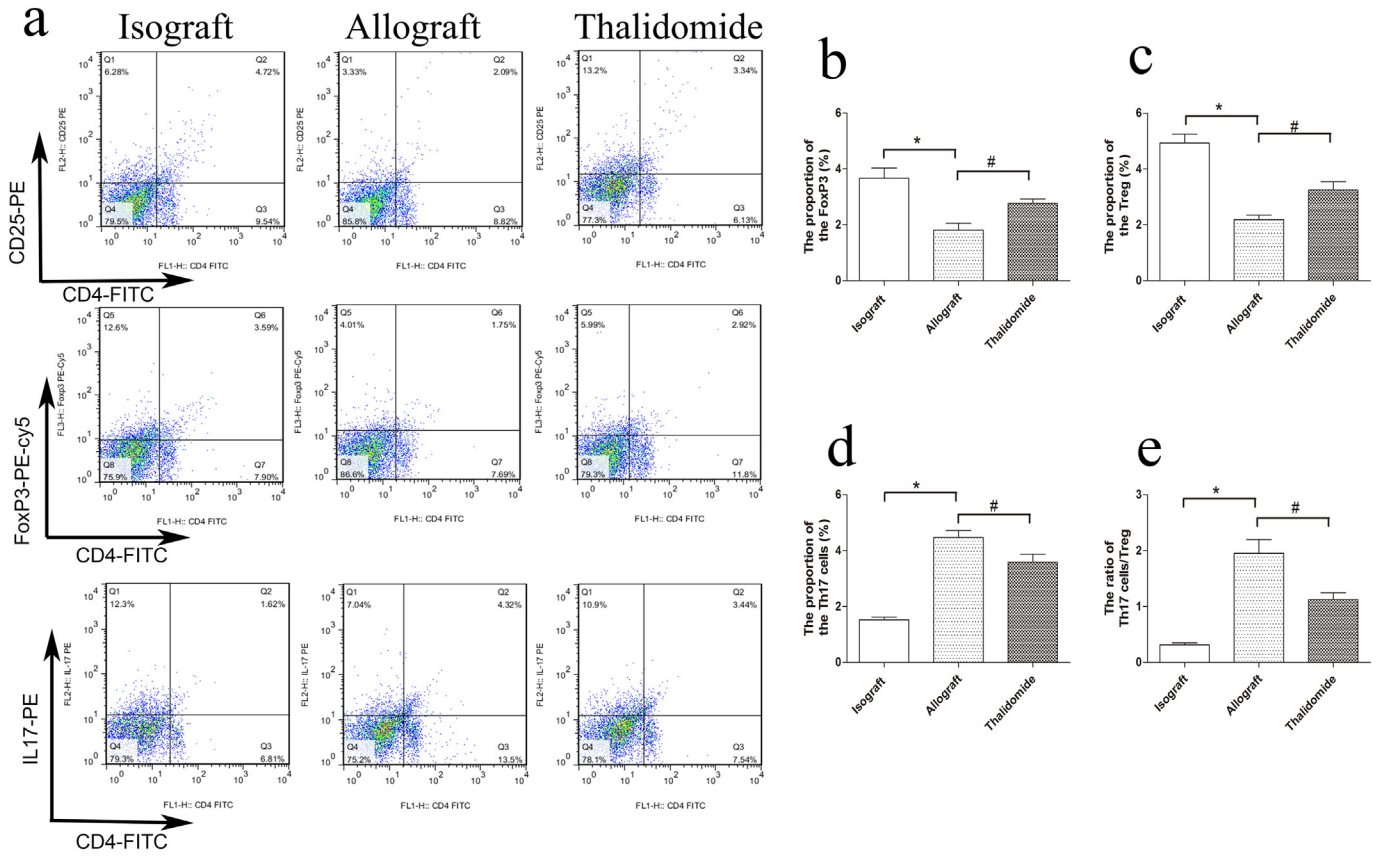

Fig. 3. (a) The proportion of the $CD4^+CD25^+$Treg, $CD4^+FoxP3^+$ and $CD4^+Th17^+$ cells in the rat peripheral blood in the different groups. (b) Thalidomide significantly increased the $CD4^+FoxP3^+$ Tregs in the peripheral blood after kidney transplantation. (c) Thalidomide significantly increased the $CD4^+CD25^+$ Tregs in the peripheral blood after kidney transplantation. (d) Thalidomide significantly decreased the CD4 + Th17+ cells in the peripheral blood after kidney transplantation. (e) Thalidomide significantly decreased the ratio of $CD4^+CD25^+$ T cells/$Th17^+$ cells after kidney transplantation. *$P < 0.05$ in comparison with isograft group. #$P < 0.05$ in comparison with allograft group. $N = 6$ for each group. The bar represents mean ± SEM.

3.4. Thalidomide moderated the distribution of the $CD4^+CD25^+$ regulatory T cells, $CD4^+FoxP3^+$ regulatory T cells (Treg) and $CD4^+Th17$ cells in the peripheral blood

$CD4^+CD25^+$ and $CD4^+FoxP3^+$ Tregs were markedly decreased in the allogeneic group compared with the isograft control group ($P < 0.05$) (Fig. 3), whereas the daily treatment with thalidomide significantly increased the $CD4^+CD25^+$ and $CD4^+FoxP3^+$ Tregs in the peripheral blood ($P < 0.05$) (Fig. 3). However, there was a significant increase in the percentage of $Th17^+$cells of the total $CD4^+$T cells obtained from the allogeneic group compared with that in the isograft control group ($p < 0.05$) (Fig. 3), whereas the percentage of Th17 cells in thalidomide recipients were significantly decreased compared with that in the allogeneic group ($P < 0.05$) (Fig. 3).

3.5. Thalidomide decreased expression of TGF-β1, α-SMA, and VEGF in the kidney graft after kidney transplantation

Immunofluorescence showed that the expression of TGF-β1, α-SMA, and VEGF in the allogeneic group was significantly increased compared with that in the isograft control group. Thalidomide significantly reduced the expression of TGF-β1, α-SMA, and VEGF in transplanted kidneys compared with that in the allogeneic group (Fig. 4a).

Western blot analyses of transplanted kidneys also revealed that the expression of TGF-β1, α-SMA, and VEGF in the allogeneic group was significantly increased compared with the isograft control group ($P < 0.05$) (Fig. 4b, c, and d, respectively). The administration of thalidomide significantly decreased the expression of TGF-β1, α-SMA and VEGF in transplanted kidneys compared with that in the allogeneic group (Fig. 4b, c, and d, respectively) ($P < 0.05$).

4. Discussion

In this study, we demonstrated for the first time that thalidomide ameliorates allograft chronic TCMR in a well-described Fischer to Lewis rat kidney transplantation model. Thalidomide treatment improved renal function and attenuated the histological changes in the kidney graft after kidney transplantation. Moreover, thalidomide treatment reduced serum concentrations of inflammatory cytokines and changed the percentage of T cell subsets in the peripheral blood. In addition, thalidomide decreased the expression of proteins related to fibrosis and neointima formation.

Despite a significant improvement in short-term graft survival and acute rejection rates over the last decades [2], long-term graft survival has not improved substantially [17,18]. Transplant chronic rejection is the major cause of renal transplant failure, and there is no effective treatment so far [19]. Chronic T cell mediated rejection (TCMR), one type of Chronic rejection, is characterized by infiltration of the interstitium by effector T cells, B cells, and myeloid cells with dendritic cell and macrophage markers [20,21]. TCMR is defined by histologic features of interstitial inflammation, tubulitis and/or intimal arteritis [22]. TCMR is an inflammation in the interstitium of allograft tissue, initiated by homologous conjugation of donor antigens with antigen presenting cells (APCs) [23,24]. The main unit of cognate recognition in TCMR is effector T cells that are bound to donor antigen on macrophages. The synaptic active effector T cells induce the production of interferon-gamma (IFNγ) [25] and activate APC. This event recruits effector memory CD4 and CD8 T cells, and macrophage precursors activate the

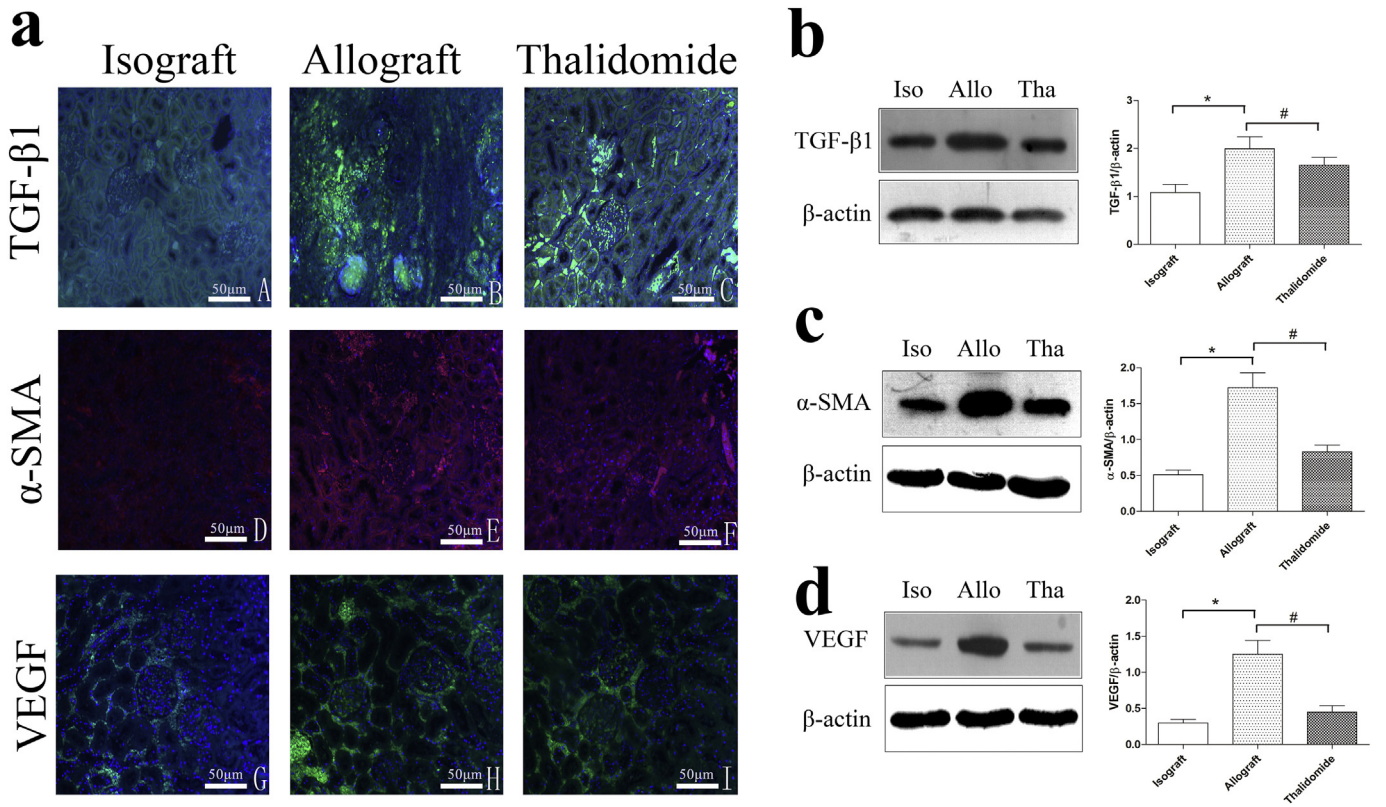

Fig. 4. (a) Thalidomide moderated expression of TGF-β1, α-SMA, and VEGF in the kidney graft after kidney transplantation. Representative micrographs of (A, B, C) TGF-β1 staining, (D, E, F) a-SMA staining, and (G, H, I) VEGF staining, Original magnification is 200×, and scale bars represent 50 μm. Western blot showed thalidomide significantly attenuated the expression of (b) TGF-β1, (c) α-SMA, and (d) VEGF in kidney graft after kidney transplantation. The same blot was stripped and re-probed with actin to confirm equal loading. $^{*}P < 0.05$ in comparison with the isograft group. $^{\#}P < 0.05$ in comparison with the allograft group. $N = 6$ for each group. The bar represents mean ± SEM.

inflammasome [26], creating an interstitial inflammatory compartment. TCMR leads to local inflammation and epithelial dedifferentiation and tubulitis [20].

Kidney biopsy in patients with chronic rejection showed arterial intimal thickening, glomerular sclerosis, interstitial fibrosis, and tubular atrophy. Interstitial fibrosis and tubular atrophy (IFTA) remain the leading cause of chronic histological damage and graft loss 5 years after transplantation [27]. Transplant vasculopathy, also known as graft arteriosclerosis, is a hallmark of chronic renal transplant rejection [28]. Although mTOR inhibitors have shown good potential in the treatment of transplant vasculopathy [29], there is no drug available for clinical use yet. Transplant vasculopathy occurs not only in the large arteries, but also in the small peritubular capillaries [30].

Our previous study has shown that the thalidomide treatment effectively attenuates transplant arteriosclerosis in aortic allograft model [14]. Another researcher also confirmed that thalidomide prevents allograft vasculopathy after aortic transplantation [15]. In 1994, thalidomide was found to inhibit the formation of new blood vessels [31]. Based on anti-angiogenesis effects, thalidomide was used to treat refractory multiple myeloma and proved to be effective [32]. Thalidomide was also found to be effective against other cancer types in subsequent clinical trials [33–36]. In this study, thalidomide treatment significantly attenuated transplant arteriosclerosis. It not only attenuated vasculopathy in the large arteries, but also affected small peritubular capillaries. Western blot analyses of transplanted kidneys showed that the thalidomide treatment significantly decreased the expression of VEGF, a crucial growth factor in the formation of new blood vessels. This may be the mechanism by which thalidomide attenuates transplant arteriosclerosis of kidney grafts.

After the withdrawal of thalidomide in the market, some studies have found that thalidomide has immunomodulatory and anti-inflammatory properties [8] and that thalidomide inhibits the production of inflammatory cytokines and reduces inflammation [37]. Meanwhile, thalidomide alters the activities and the secretion of various cytokines [38–40]. Some studies have demonstrated that thalidomide has antifibrotic effects in multiple myeloma or myelofibrosis and pulmonary fibrosis [7,41,42]. In our study, thalidomide treatment significantly decreased the levels of both proinflammatory cytokines and inflammatory cytokine. Therefore, we can conjecture that thalidomide may suppress the induction of chronic rejection and be beneficial to long-term allograft survival.

Thalidomide has multiple effects on the immune system and the cytokine cascade [43]. Thalidomide was first shown to act in graft-versus-host disease (GVHD) [44]. Subsequent data indicated that it can be used as a therapeutic agent for acute [45] and chronic GVHD after stem cell transplantation [12,46,47].

As early as 1970, Murphy et al. used thalidomide as an immunosuppressant in a canine renal allotransplantation [48]. Subsequent studies have confirmed that thalidomide is an effective alternative to corticosteroids after lung transplantation [49] and heart transplantation in rat models [50]. Recently, thalidomide was used as an adjuvant immunosuppressive drug to improve heart and skin allograft survival in heterotopic heart transplantation [13,51] and skin transplantation models [52,53]. Furthermore, thalidomide improves islet functions and grafts survival in pancreatic islet xenotransplantation models [54,55]. In the present study, thalidomide treatment significantly reduced SCr levels after kidney transplantation. Histological examination showed that thalidomide significantly ameliorated vasculopathy, glomerulopathy, interstitial fibrosis, mononuclear cell infiltration, and tubular atrophy, particularly intimal proliferation of the

grafted arteries. The Masson's trichrome stain also showed that the daily treatment with thalidomide significantly suppressed the tubulointerstitial collagen deposition. Histological analysis showed a lower Banff score in the rats treated daily with thalidomide compared to those only subjected to allogeneic kidney transplantation. Taken together, these data suggest that thalidomide can reduce tissue inflammation and damage, alleviate chronic rejection, and eventually protect kidney allografts.

It is well known that Th17/Treg balance plays a key role in the control of allograft rejection [56,57]. Th17 cells regulated inflammation via the production of IL-17, such that the level of IL-17A increased in immune rejection [58]. IL-17A inhibition significantly prolonged allograft survival [59]. Treg cells have been regarded as a viable alternative to control the immune reactivity of solid organ allografts and induced immunological tolerance in clinical transplantation [60–63]. As the proportion of Th17/Treg cells increases, autoimmune and proinflammatory effects appear to predominate. In contrast, immune tolerance and anti-inflammatory effects predominate as this ratio decreases. Therefore, manipulating the balance of Th17/Treg is a method of preventing allograft rejection.

Interestingly, the number of $CD4^+CD25^+$ and $CD4^+FoxP3^+$Tregs in the peripheral blood in the thalidomide-treated rats was significantly higher than that in the Allo control group, while the number of Th17 cells was markedly lower than that in the Allo control group ($P < 0.05$). Furthermore, the ratio of Th17/Treg cells in the thalidomide treated rats was significantly lower than that in the Allo control group ($P < 0.05$). This coincides with the previous research that thalidomide treatment decreased IL-17 level during allergic asthma [64] and upregulated $CD4^+FoxP3^+$Tregs in vitro [65–67]. The results of this study suggest that thalidomide may alleviate allograft rejection by regulating the Th17/Treg balance.

In conclusion, our study demonstrated that thalidomide treatment alleviates graft chronic TCMR in an allogeneic kidney transplant model. Thalidomide has prominent potential as a basic immunosuppressive drug, the detailed mechanism of which requires further research.

Conflict of interest statement

None.

Acknowledgments

This work was supported by Zhejiang Provincial Natural Science Foundation of China under Grant No. LQ16H100002, LY16H050007; Zhejiang Provincial Science Technology Foundation 2015C33186.

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