

Leflunomide Inhibits rat-to-Mouse Cardiac Xenograft Rejection by Suppressing Adaptive Immune Cell Response and NF- κ B Signaling Activation

Cell Transplantation
Volume 30: 1–11
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DOI: 10.1177/09636897211054503
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Abstract

Xenotransplantation is a potential solution for the severe shortage of human donor organs and tissues. The generation of humanized animal models attenuates strong innate immune responses, such as complement-mediated hyperacute rejection. However, acute vascular rejection and cell mediated rejection remain primary barriers to xenotransplantation, which limits its clinical application. In this study, we systematically investigated the immunosuppressive effect of LEF using a rat-to-mouse heart xenotransplantation model. SD rat xenogeneic hearts were transplanted into C57BL/6 mice, and survived 34.5 days after LEF treatment. In contrast, BALB/c allogeneic hearts were transplanted into C57BL/6 mice, and survived 31 days after LEF treatment. Compared to normal saline treatment, LEF treatment decreased xenoreactive T cells and CD19⁺ B cells in recipient splenocytes. Most importantly, LEF treatment protected myocardial cells by decreasing xenoreactive T and B cell infiltration, inflammatory gene expression, and IgM deposition in grafts. In vivo assays revealed that LEF treatment eliminated xenoreactive and alloreactive T and B lymphocytes by suppressing the activation of the NF- κ B signaling pathway. Taken together, these observations complement the evidence supporting the potential use of LEF in xenotransplantation.

Keywords

xenotransplantation, heart transplant, T cell biology and B cell biology

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Submitted: April 19, 2021. Revised: September 21, 2021. Accepted: October 4, 2021.

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Introduction

Xenotransplantation may offer a great source of tissues and organs to patients who need transplantation urgently, contributing to the potential solving of the current severe shortage of human donors. The development of gene editing technologies has made the development of humanized animal models more convenient, promoting the progression of xenotransplantation applications^{1,2} and attenuating strong innate immune responses, such as complement-mediated hyperacute rejection³. However, even in cases where hyperacute rejection is prevented⁴, other mechanisms such as adaptive T-cell-dependent or -independent acute vascular rejection (AVR)⁵ and cell-mediated rejection (CMR)⁶, can lead to aggressive xenograft rejection^{7,8}.

Due to the explicit genetic background, immune response mechanism, and various gene-editing strains of mice, the rat-to-mouse organ xenotransplantation model has become an essential tool for studying the mechanism of xenograft rejection. Rat hearts transplanted into naive C57BL/6 mice do not encounter hyperacute rejection. However, they are rejected within 20 days after transplantation, accompanied by T cell infiltrates and an AVR profile⁹.

Leflunomide (LEF) exhibits immunosuppressive effect in inhibiting T cell activation and proliferation, as well as the ability of B cells in secretion and proliferation of immunoglobulins^{10–12}. Biochemical analyses have revealed that the mechanism of immunosuppression by LEF involves at least two separate activities: inhibition of de novo pyrimidine synthesis, which results in inhibition of cell proliferation, and inhibition of tyrosine phosphorylation, which results in inhibition of cell activation^{13,14}. Thus, LEF has the potential to inhibit both T and B cell functions. However, *in vivo* experiments have demonstrated the opposite results¹⁵. In a mouse-to-rat heart transplantation model¹⁶, LEF monotherapy showed no effect on the production of Th1 cytokines and T cell function. Conversely, in a hamster-to-rat heart transplantation model, LEF successfully prolonged concordant xenograft survival by reducing peripheral donor-specific antibodies and the proportion of CD4⁺ T cells¹⁷. Therefore, our study intended to verify the immunoregulation mechanism of LEF in a concordant xenotransplantation model of rat-to-mouse heart transplantation, and to provide evidence for experimental organ xenotransplantation. Simultaneously, a parallel experiment was performed with LEF in a mouse-to-mouse allograft model to investigate the efficacy of LEF in cardiac allotransplantation and xenotransplantation.

Materials and Methods

Animals & Drugs

SD rats (male, 20–23 g) and BALB/c and C57BL/6 mice (male, 22–25 g) were purchased from SLAC Laboratory Animal Co. Ltd (Shanghai, China), and bred in a pathogen-free facility at

25 ± 2°C with 4–5 mice per cage. All care and handling of animals was conducted in accordance with the guidelines of the Animal Care and Use Committee and Ethics Committee of Xiamen University (Committee's reference number: XMULAC20170243). LEF was purchased from Selleck (Shanghai, China) and dissolved in 1% carboxymethylcellulose to obtain a final concentration of 5 mg/mL.

Heart Transplantation & Treatment

SD rats and BALB/c mice were used as donors, and C57BL/6 mice were used as recipients. Donor hearts were transplanted heterotopically into the recipient mice with anastomosis to the vessels of the neck using a non-suture cuff technique^{18–20}. After the procedure, LEF was administered to the recipient mice at 30 mg/(kg·d) intraperitoneally in the LEF group. Normal saline treatment was administered to the control group. Graft survival was assessed by palpation once a day until the last complete graft rejection (defined as the loss of palpable cardiac contractions).

Pathology Assay

The xenograft and allograft were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and cut into 5 µm sections for hematoxylin and eosin (H&E) staining. Immunohistochemical staining was performed as described previously¹⁷, the sections were then stained with primary antibody anti-CD4 Ab (Boster, Wuhan, China), anti-CD8 Ab (Boster), anti-CD20 Ab (Boster), anti-IgM (Boster), and secondary antibody goat anti-rabbit IgG (GB23303; Boster, Wuhan, China). Samples were visualized with a DAB detection kit (Maixin-Bio, Fuzhou, China). We used a pathological section scanner (Pannoramic P250, 3DHISTECH, Budapest, Hungary) to analyze the immunohistochemical staining density. Two cardiologists blinded to the experimental conditions graded acute rejection according to the International Society of Heart and Lung Transplantation (ISHLT) criteria²¹. Briefly, 0 R = no rejection; 1 R (mild rejection) = evidence of perivascular infiltrate, interstitial infiltrate, or both with up to 1 focus of myocyte damage; 2 R (moderate rejection) = two or more infiltrate foci with related myocyte damage; 3 R (severe rejection) = the infiltrate was diffuse and had multifocal myocyte damage ± edema, ± hemorrhage, ± vasculitis.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

mRNA was isolated from the heart grafts at POD 6 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a ReverTra Ace[®] qPCR RT Kit (code no. FSQ-101, Toyobo, Kyoto, Japan) followed by quantitative real-time PCR (qRT-PCR) using SYBR Green Real Time PCR Master Mix -Plus (code no. QPK-212, 212 T, Toyobo, Kyoto, Japan). All the protocols were performed according

to the manufacturer's instructions. β -actin was used as an internal control. All reactions were performed in triplicate.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum from the recipient mice were collected at POD 6. The presence of IFN- γ was investigated using commercially available ELISA kits (Yikesai Bioproduct Limited Company, Shanghai, China) according to the manufacturer's instructions. Each reaction was performed in triplicate. A standard curve was constructed using known amounts of purified recombinant murine cytokines.

Flow Cytometry

One million splenocytes from naïve SD and BALB/c were used as target cells to measure antibodies in the recipient serum as indicated for reactivity measurements. After incubation for 30 min at 4°C, anti-mouse secondary antibodies fluorescein isothiocyanate (FITC)-anti-IgG1 (eBioscience, San Diego, CA, USA), phycoerythrin (PE)-anti-IgM (eBioscience), and FITC-anti-IgG2a (eBioscience) were added for further incubation at 4°C. Splenocytes (1×10^6) from the recipient animals were incubated with APC-anti-CD3 (eBioscience), FITC-anti-CD4 (eBioscience), PE-anti-CD8 (eBioscience) and their respective isotype controls for 30 min. Regulatory T cells were stained using a commercial kit (eBioscience) according to the manufacturer's instructions. In both cases, cells were washed with PBS and analyzed by flow cytometry (Beckman Coulter Gallios™, Kaluza® Analysis Software). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Mixed Lymphocyte Reaction (MLR)

The MLR is a one-way stimulation by treating the cells of one individual with mitomycin C, which inhibits DNA synthesis²². Splenocytes from naïve BALB/c mice or SD rats treated with mitomycin C (40 μ g/mL, Dalian Meilun Biotechnology Co., LTD, Dalian, China) were used as stimulator cells, whereas splenocytes from the recipient C57BL/6 mice were used as the responder cells. The stimulator and responder cells (ratio 1:10) were added to a 96-well round-bottom plate and cultured at 37°C for 72 h. Cell proliferation was measured using the BrdU kit (Roche Diagnostics, Indianapolis, IN, USA). Each experiment was performed in triplicate.

Protein Extraction and Western Blotting (WB)

Splenocytes were lysed in fresh extraction buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with a protease inhibitor and phosphatase inhibitor (Gold Biotechnology, St. Louis, MO, USA). The extracted proteins (20 μ g) were separated on a 15% SDS-polyacrylamide gel and electroblotted onto a polyvinylidene fluoride membrane. The membrane was blocked using 5% milk dissolved in TBST for 60 min at 20–25°C and incubated overnight at 4°C with primary

antibodies against p-p38 (AF4001, Affinity Biosciences, Changzhou, China), p38 (AF6456, Affinity Biosciences) p-p65 (AF2006, Affinity Biosciences), p65 (AF5006, Affinity Biosciences), p-I κ B α (AF2002, Affinity Biosciences) and I κ B α (AF5002, Affinity Biosciences). The membranes were then washed with TBST and incubated with goat anti-mouse IgG (1:1000; HAF007, R&D Systems, Inc., Minneapolis, MN, USA) and goat anti-rabbit IgG (1:1000; HAF008, R&D Systems, Inc., Minneapolis, MN, USA). Bound antibodies were detected using an electrochemiluminescence detection system (Amersham Life Science, Arlington Heights, IL, USA). β -Actin was used as the control and to ensure equal protein loading.

Statistical Analysis

The Kaplan–Meier method was used to calculate the mean survival time of grafts in each group. The Mann–Whitney U test was applied to compare rejection/inflammatory events. Mixed lymphocyte reaction assays, flow cytometric analysis, ELISA, and qRT-PCR data were analyzed using one-way analysis of variance (ANOVA). Student's *t*-test was used to analyze statistically significant differences in the designed data. Bonferroni correction was calculated and applied because of multiple comparisons. A value of $P < 0.05$ was considered statistically significant. All analyses were performed using the GraphPad Prism (GraphPad, Inc., La Jolla, CA, USA) software.

Results

LEF Significantly Prolongs Graft Survival in Concordant Xenogeneic and Allogeneic Heart Transplantation

To determine the therapeutic effect of LEF on concordant xenogeneic heart transplantation model, SD rats were used as donors, C57BL/6 mice were used as recipients, and cervical heterotopic heart transplantation was performed. The kinetics of the xenograft survival rates for all the study groups are shown in Fig. 1A. All xenografts in the normal saline-treated groups were rejected within 15 days after transplantation, with a media graft survival time of 11.5 days. Treatment with low dosage (15mg/kg.d) of LEF therapy, moderately extended median xenograft survival time to 20.5 days, and median allograft survival time to 18 days. Treatment with moderate dosage of LEF significantly prolonged xenograft survival to 34.5 days. All cardiac xenografts in the LEF-treated group developed rejection within 48 days. The regimen was designed to achieve a similar duration of treatment in the BALB/c to C57BL/6 cardiac allograft model (Fig. 1B). In comparison with the normal saline treatment group (MST=7 days), there was a significant extension in survival time of the allograft after low dosage (MST=18 days) and moderate dosage (MST=31 days) LEF treatment. However, there was no markedly difference between the survival times of the

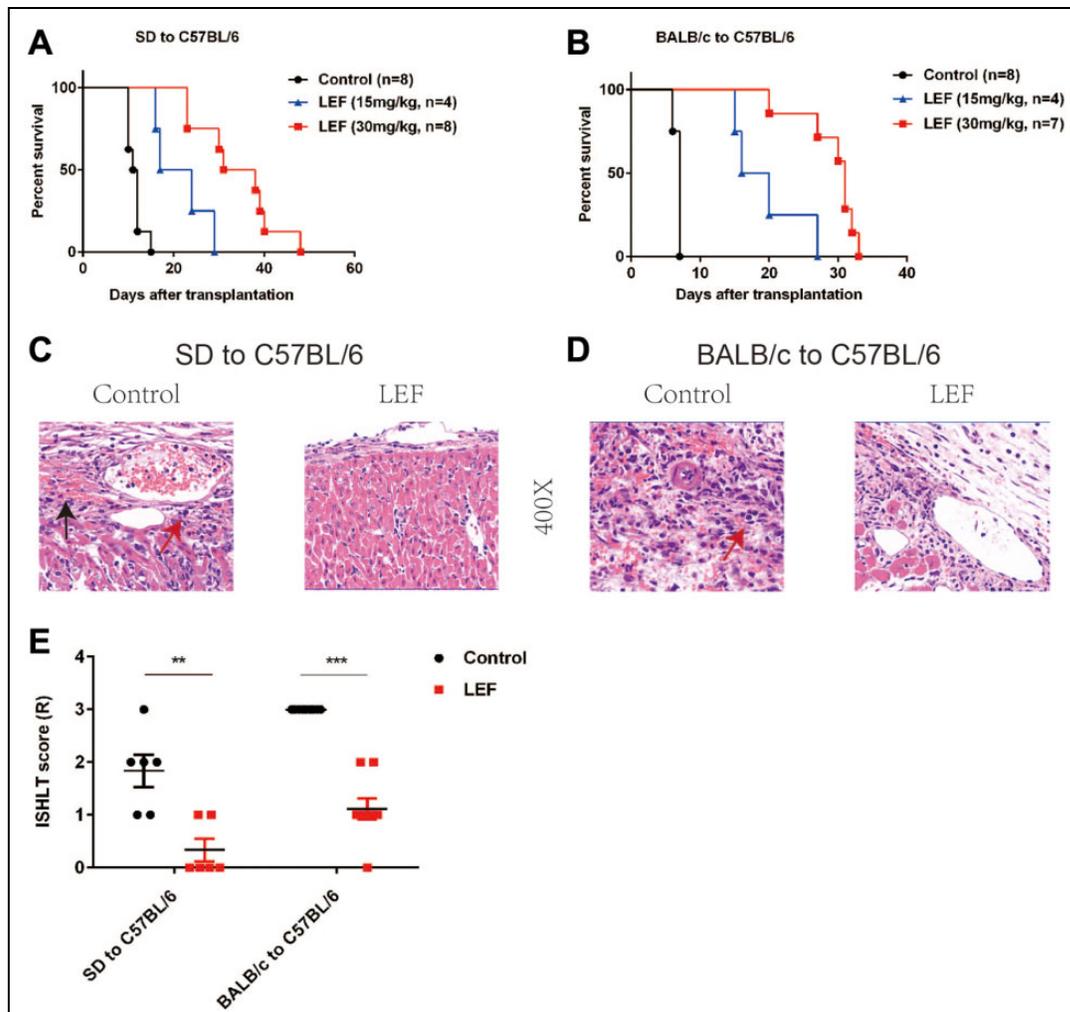


Figure 1. The effect of LEF on xenogeneic or allogeneic heart transplantation. SD or BALB/c heart grafts were transplanted into C57BL/6 mice at POD 0. Recipients were treated with normal saline, 15 mg/(kg·d) and 30 mg/(kg·d) of LEF intraperitoneally (i.p.) at POD 0. Survival time of heart (A) xenografts and (B) allografts. H&E staining of heart (C) xenografts and (D) allografts collected at POD 6 from normal saline-treated and LEF-treated (30 mg/(kg·d)) mice recipients (original magnification: $\times 400$). (E) International society for heart and lung transplantation (ISHLT) grades from nine different sections from three heart xenografts and allografts in each group. H&E = hematoxylin and eosin; POD = postoperative day; Data are presented as mean \pm SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the normal saline-treated group.

xenograft and allograft after moderate LEF treatment. We also tested the efficacy of the LEF at 40 mg/kg, all recipient mice experienced perioperative deaths (data not shown). Thus, moderate dosage of LEF was used for the follow up study.

H&E staining was performed to assess the pathological changes associated with acute xenograft rejection. Xenografts in the normal saline-treated group predominantly demonstrated AVR and CMR at postoperative day (POD) 6, characterized by massive interstitial hemorrhage (black arrow, Fig. 1C), and moderate mononuclear cell infiltration (red arrow, Fig. 1C). In contrast, allografts in the normal saline-treated group predominantly developed CMR at POD 6, characterized by mononuclear cell infiltration and tissue injury (Fig. 1D). Recipients treated with LEF demonstrated significant attenuation of these pathological changes in

xenografts and allografts at POD 6, characterized by lower ISHLT rejection score (Fig. 1E), although AVR and CMR rejection eventually developed by the endpoint.

Treatment with LEF Suppresses Xenoreactive T and B Cell Immune Responses

To evaluate the immunosuppressive activity of LEF in recipient mice after heart transplantation, recipients' splenocytes were harvested and incubated with irradiated SD splenocytes. This assay demonstrated that LEF treatment significantly reduced splenocyte proliferative responses to xeno-antigens compared to normal saline treatment, and a similar inhibiting effect on allo-antigens of LEF was observed in the allogeneic transplantation model (Fig. 2A).

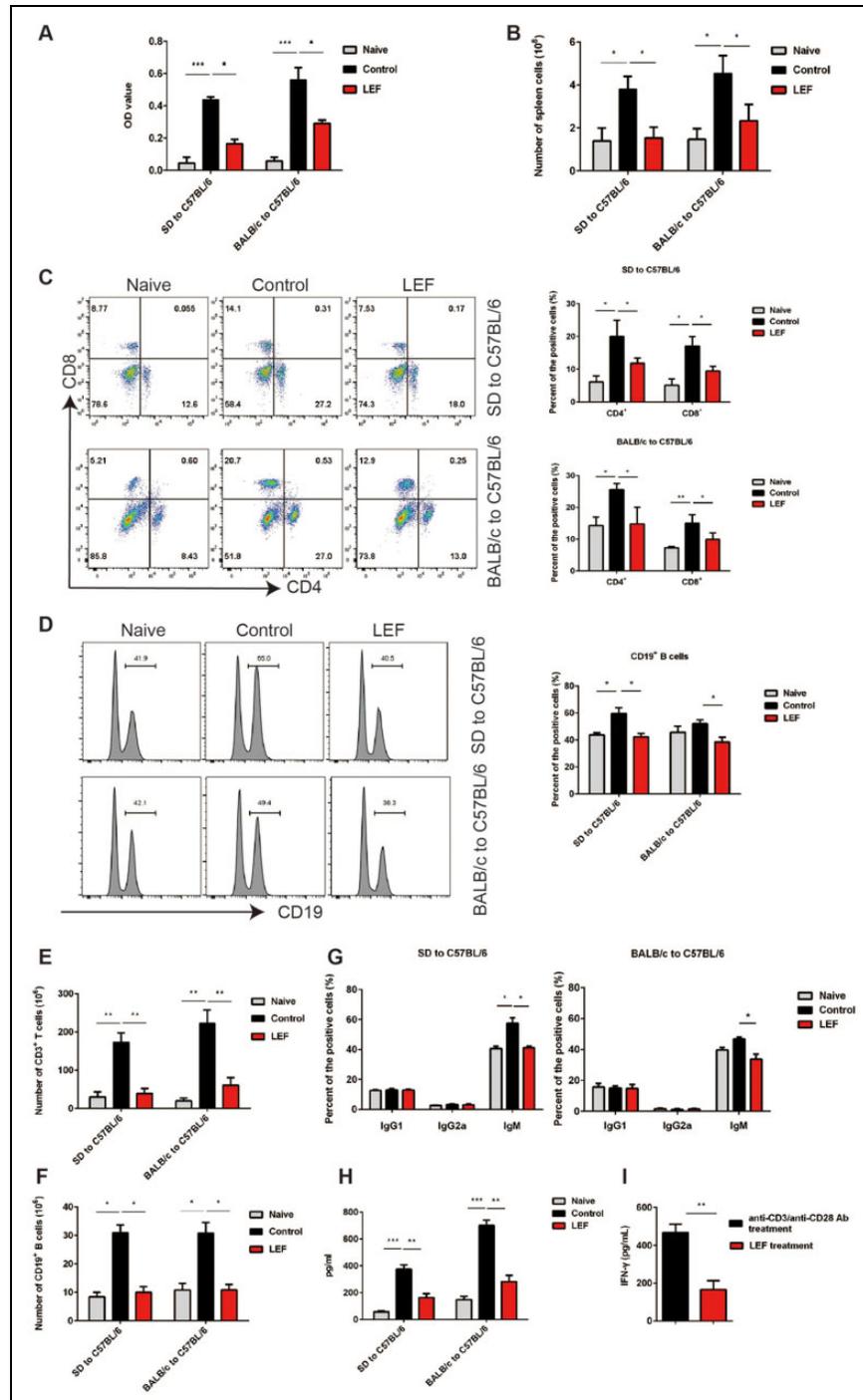


Figure 2. The effect of LEF treatment on CMR- and AVR- mediated immune responses. (A) MLR responses. Recipient splenocytes were isolated at POD 6 (responders) and irradiated naïve SD or BALB/c splenocytes (stimulators) were co-cultured for three days. Data are representative of three independent experiments. (B) Absolute numbers of splenocytes in LEF -treated and normal-saline treated mice recipients. Naïve C57BL/6 mice are shown for comparison ($n = 3$ mice/group). (C) Representative proportion of CD4⁺ and CD8⁺ T cells in recipient splenocytes. A total of 1×10^6 splenocytes were isolated at POD 6, and the percentage of CD4⁺ and CD8⁺ T cells was determined by flow cytometry ($n = 3$ mice/group). (D) Representative proportion of CD19⁺ B cells in recipient splenocytes. A total of 1×10^6 splenocytes were isolated at POD 6, and the percentage of CD19⁺ B cells was determined by flow cytometry ($n = 3$ mice/group). (E) The number of CD3⁺ T cells in recipient spleen were determined by flow cytometry ($n = 3$ mice/group). (F) The number of CD19⁺ B cells in recipient spleen were determined by flow cytometry ($n = 3$ mice/group). (G) Representative proportion of donor-specific antibodies in recipient serum. Serum was collected from xenograft and allograft recipients at POD 6, and the percent of IgG1, IgG2a, and IgM was determined by flow cytometry ($n = 3$ mice/group). (H) Serum levels of proinflammatory cytokines. Peripheral blood was collected at POD 6 and IFN- γ serum levels were measured by ELISA ($n = 3$ mice/group). (I) CD3⁺ T cells were isolated from naïve C57BL/6 mice, and co-cultured with anti-CD3 and anti-CD28 monoclonal antibody in the absence and presence of LEF for 3 days, the supernatant was collected and measured by ELISA ($n = 3$ separate experiments). Data are presented as the mean \pm SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the normal saline-treated group.

Compared to normal saline treatment, the absolute number of splenocytes was significantly lower in LEF-treated mice, but similar to that in naïve mice (Fig. 2B). Flow cytometric analysis revealed lower percent of both CD4⁺ and CD8⁺ T cells from spleens of LEF-treated xenograft (Fig. 2C) and allograft (Fig. 2C) recipient mice at POD 6 when compared to those from normal saline-treated recipients. Xenograft recipients in the control group demonstrated a higher percentage of CD19⁺ B cells than recipient administration of LEF (Fig. 2D). These differences were not observed in the allograft recipients. We also evaluated the absolute number of CD3⁺ T and CD19⁺ B cells in recipient mice spleen at POD 6. Compared to normal saline treatment, the number of CD3⁺ T cells were significantly lower in LEF-treated mice but similar to naïve mice ($P < 0.05$, Fig. 2E). Similar results were obtained when investigating the number of CD19⁺ B cells in xenotransplantation and allotransplantation (Fig. 2F). The circulating anti-rat IgG1, IgG2a, and IgM in the recipients' sera were also evaluated by flow cytometry using SD splenocytes as target cells. Both anti-rat IgG1 and IgG2a levels did not significantly change in the sera between normal saline and LEF-treated mice. IgM levels were significantly elevated in the sera of normal saline-treated mice at POD 6. In contrast, LEF treatment significantly reduced the levels of IgM antibodies (Fig. 2G). To evaluate whether LEF regulates the production of proinflammatory cytokines, transplant recipient serum was collected at POD 6, and IFN- γ protein secretion was measured by ELISA (Fig. 2H). Decreased levels of serum proinflammatory cytokine IFN- γ were observed in the LEF-treated recipients compared to those treated with normal saline ($p < 0.01$). We further determined whether LEF would inhibit T cells function in vitro. FACS-sorted CD3⁺ T cells from naïve C57BL/6 mice, co-cultured with anti-CD3 and anti-CD28 monoclonal antibody in the absence or presence of LEF for 3 days. The supernatant was collected and the protein level of IFN- γ was measured by ELISA. We found that LEF significantly inhibited IFN- γ generation by CD3⁺ T cells ($P < 0.01$, Fig. 2I).

Treatment with LEF Protects Grafts

In order to explore the possible anti-rejection mechanism of LEF involved in heart graft protection and destruction, a series of cytolytic and effector genes expressed in xenografts were assessed at POD 6. LEF treatment led to reduced expression of IL-2, IFN- γ , IL-4, and TNF- α mRNA as compared to normal saline treatment, suggesting the downregulation of Th1 and Th2 cell responses (Fig. 3A). However, the Th2 cell response was not involved in allograft, which was reflected in the non-significantly different expression of IL-4 mRNA between LEF or normal saline treatment (Fig. 3B).

The process of AVR- and CMR-mediated xenograft rejection is associated with antibody deposition and mononuclear cell infiltration. These findings were supported by immunohistochemistry results: anti-CD20⁺ B cells (Fig. 3C) and anti-rat IgM (black arrow, Fig. 3D) deposition in the

xenograft was significantly reduced in the LEF-treated group compared to the normal saline-treated group at POD 6 as well as in allotransplantation. At this point, LEF-treated mice showed fewer CD4⁺ and CD8⁺ T cells in normal myocardial fibers compared with normal saline-treated mice (Fig. 3E). Alloreactive T cells were also decreased in allografts of LEF treatment (Fig. 3F). This observation indicated that LEF has the capacity to suppress xenoreactive T cell responses and delay the generation of xenoreactive antibodies.

Treatment with LEF inhibits the Activation of the Nuclear Factor-Kappa B (NF- κ B) Signaling Pathway

An in vitro assay had demonstrated that LEF inhibits lymphocyte generation by blocking NF- κ B signaling activation²³. We determined whether LEF would have an effect on xenoreactive and alloreactive T and B cells NF- κ B signaling. In our study, the expression of phospho-I κ B α and phospho-p65 in recipient splenocytes was measured using WB analysis at POD 6. LEF significantly inhibited the expression of phospho-I κ B α and phospho-p65 compared to that in the normal saline-treated group. Results of the present investigation showed that the mitogen-activated protein kinases (MAPK) signaling pathway partly facilitated the activation of NF- κ B signaling pathway, leading to the response to inflammatory stimulus²⁴. We measured the phosphorylation level of the stress responsive MAPK, such as p38 MAPK. The phosphorylation level of p-p38 induced by xenoantigen or alloantigen in the control mice was significantly increase compare to LEF treatment (Fig. 4). In this study, the anti-inflammatory effect of LEF was proved through its ability to decrease the activity of p38 MAPK and NF- κ B.

Discussion

Xenotransplantation may be a potential solution to the shortage of human donor organs. Genetically modified techniques eliminate the hyperacute rejection barrier²⁵; however, xenogeneic organs undergo CMR and AVR within days after transplantation. Subsequent studies indicated that the pig-to-non-human primate model is the ideal model for studying xenotransplantation²⁶. However, the cost of non-human primates and complex ethical issues limited its application. In the present study, we chose a rodent xenotransplant model with predominant CMR and AVR profiles. It has been reported that BALB/c and C57BL/6 mice reject rat cardiac xenografts with significantly different kinetics⁹. Rat hearts transplanted into C57BL/6 mice show CMR and AVR profiles. In contrast, rat cardiac xenografts in BALB/c mice show a typical AVR profile⁹. Therefore, we chose SD to C57BL/6 xenotransplantation model in our study and examined the effects of LEF on xenograft survival-related mechanisms.

LEF is a potent immunosuppressive agent in xenotransplantation models, including heart²⁷ and islet²⁸

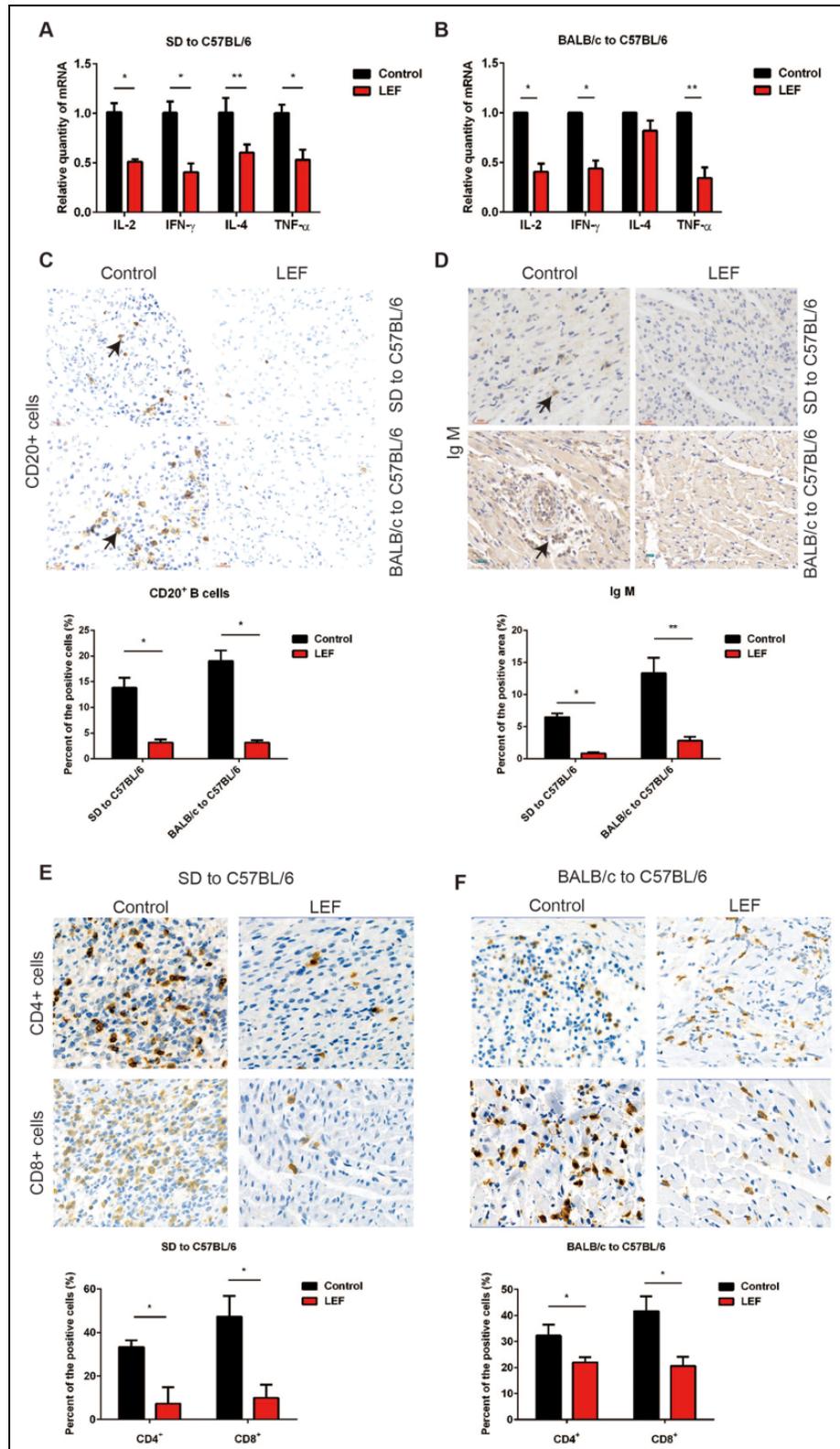


Figure 3. Phenotypic characteristics of grafts. Xenografts and allografts were recovered at POD 6. Relative mRNA expression of IL-2, IFN- γ , IL-4, and TNF- α in (A) xenograft and (B) allografts measured by qRT-PCR ($n = 3$ mice/group). (C) Immunohistochemistry staining of CD20⁺ (brown) in xenograft and allograft; bar indicates 20 μ m and quantified. (D) IgM in grafts were examined by immunohistochemistry staining; bar indicates 20 μ m and quantified. Immunohistochemistry staining of CD4⁺ (brown), and CD8⁺ (brown) in (E) xenograft and (H) allografts. (Original magnification: $\times 400$). Data are presented as mean \pm SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared to the normal saline-treated group.

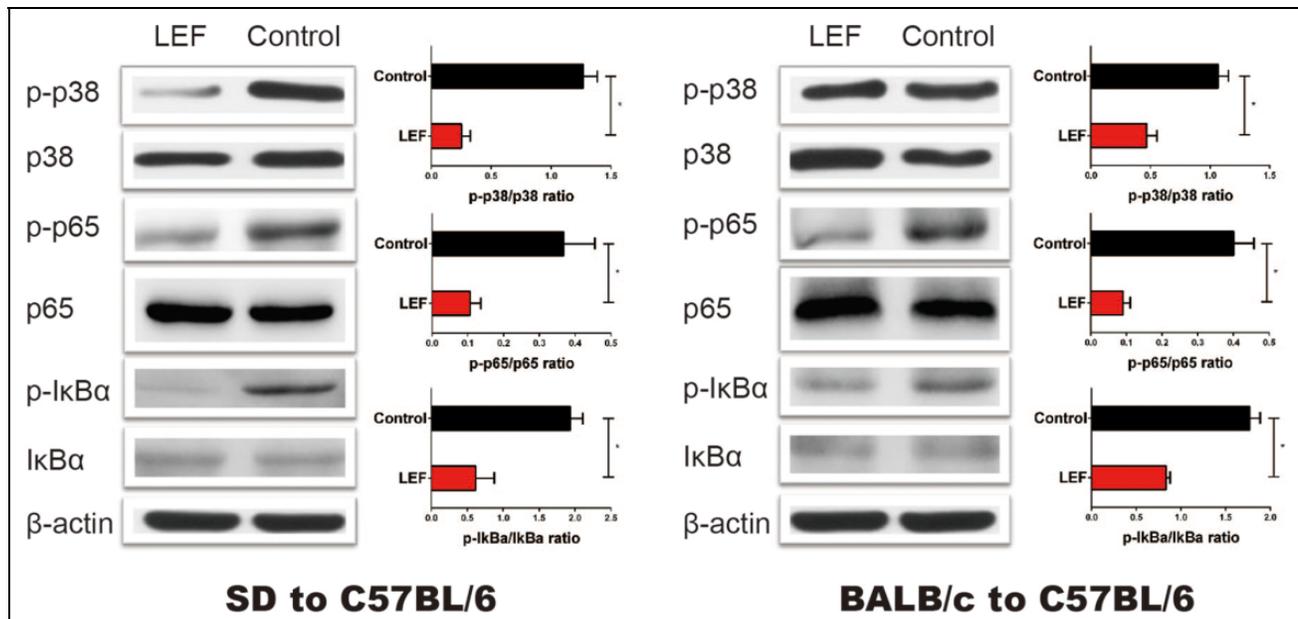


Figure 4. LEF treatment inhibited NF- κ B signaling in recipient splenocytes of the xenotransplantation and allotransplantation model. Relative protein expression of p-p38, p38, p-p65, p65, p-IkBa and IkBa in splenocytes. β -actin was used as a loading control ($n = 3$ mice/group). * $P < 0.05$ compared to the normal saline-treated group.

xenotransplantation. It has been reported that LEF can successfully attenuate AVR and prolong concordant xenograft heart survival in hamster-to-rat¹⁷, mouse-to-rat¹⁶ and hamster-to-rat²⁹ heart transplantation. In the present study, we tested the ability of LEF to inhibit xenograft rejection in a rat-to-mouse cardiac transplant model. To our knowledge, this is the first study that reported that LEF can efficiently prolong rat heart survival in mouse recipients. Our results indicated that LEF monotherapy significantly prolonged xenograft survival from 23 to 48 days, compared with normal saline-treated animals. In the same immunotherapy regimen, we observed that LEF has a better effect on prolongation of xenografts compared to allografts. LEF demonstrated similar immunomodulatory effects in protecting xenografts and allografts by decreasing inflammatory cell infiltration and tissue damage. Despite current strategies, Xenotransplantation tolerance remain a challenge in clinical practice, LEF used combined with other immunosuppressants may be a potential candidate for inducing long-term xenograft survival.

It has been reported previously that rejection of rat heart xenografts is an immune response that is closely associated with the synergistic effect of T and B cells⁹. Among the T cells, CD4⁺ and CD8⁺ T cells, which are activated in the secondary lymphoid organs and migrate to the graft, influence the process of graft rejection by producing high levels of proinflammatory cytokines, such as IFN- γ ³⁰. Early experiments suggest that LEF blocks T cell proliferation *in vitro*³¹. According to the findings of the present study, we believe that LEF significantly inhibited CMR by decreasing the proportion of CD4⁺ and CD8⁺ T cells in the recipient

spleen and reducing the number of graft-infiltrating CD4⁺ and CD8⁺ T cells in xenotransplantation and allotransplantation models. AVR is a severe pattern of rejection seen in both concordant and discordant xenograft models³². It has been reported that B cell-mediated production of IgG and IgM contributes to the rejection of xenografts⁵. We found that LEF significantly inhibited the xenoreactive B cell response, as evidenced by the delayed generation of anti-rat xenoreactive antibodies and reduced intragraft deposition of anti-rat IgM when compared to the normal saline treatment. This result was consistent with the fact that LEF can inhibit B cell proliferation and differentiation by interfering with pyrimidine metabolism and by blocking some tyrosine kinases³³. Taken together, these results suggested that LEF has some inhibitory effect on xenoreactive T and B cell responses.

Induction of xenograft tolerance is the ultimate goal of organ transplantation. However, the LEF monotherapy regimen cannot induce xenograft and allograft tolerance. The development of tolerance to grafts is associated with the levels of CD4⁺Foxp3⁺ Tregs³⁴ and Th1/Th2 balance³⁵. In our study, flow cytometric analysis showed no significant change in CD4⁺Foxp3⁺ Tregs in recipient spleen after LEF therapy (data not shown), which was consistent with the reports that LEF cannot induce CD4⁺Foxp3⁺ Treg generation^{15,16} in xenotransplantation. In addition, the influence of LEF on Th1 (IFN- γ) and Th2 cells (IL-4) was determined in xenografts. LEF monotherapy decreased the mRNA expression of IL-2, IFN- γ , and IL-4. These findings indicated that LEF treatment inhibited the differentiation of Th1/Th2 cells. We also found that LEF treatment affected the function of

T cells in Th1 (IFN- γ) generation in vitro. LEF in combination with other immunosuppressive agents may be an effective therapeutic schedule to induce xenograft tolerance. It has been proven that lower dosage of LEF in combination with other immunosuppressive agents prolong the survival of xenograft more effectively than any monotherapy. Some immunosuppressive agents, such as IL-33, has been found to expand Tregs^{36,37}, which can supplement the Tregs deficiency treated by LEF. The synergistic effect of IL-33 and LEF in rat to mice xenotransplantation should be further study.

NF- κ B, a transcription factor, plays a critical role in immune regulation and can be activated by tumor necrosis factor (TNF)^{23,31}. Activation of NF- κ B and its dependent genes have been associated with autoimmune diseases and transplant rejection. It has been reported that LEF inhibits cell proliferation by inhibiting NF- κ B and gene expression stimulated by TNF, phorbol myristate acetate and other inflammatory agents in vitro^{11,31}. Our results indicated that LEF inhibits NF- κ B activation by xenantigen in vivo. LEF blocked the phosphorylation and degradation of I κ B α and subsequent nuclear translocation of the p65 subunit, steps essential for NF- κ B activation.

In conclusion, our findings showed that LEF prolonged organ xenograft and allograft survival, decreased xenoreactive and alloreactive T and B cell infiltration of the graft, and inhibited T cell proinflammatory cytokine production (IFN γ) following transplantation. LEF also suppressed T and B cell proliferation in vivo, while inhibiting NF- κ B activation. LEF is an effective immunosuppressive compound against CMR and AVR rejection.

Abbreviations

AVR, acute vascular rejection; CMR, cell-mediated rejection; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin-eosin; IL, interleukin; LEF, leflunomide; MLR, mixed lymphocyte reaction; MST, median survival time; NF- κ B, nuclear factor-kappa B; POD, postoperative day; qRT-PCR, quantitative real-time PCR; TNF, tumor necrosis factor; WB, western blotting

Acknowledgment

We thank Haiping Zheng and Xiang You for flow cytometry technical support.

Authors' Contributions

B.X. and Z.Q. conceived and designed the experiments. Y.M., M.Z., Y.C., J.H., L.Z., Q.L., and J.Z. performed the experiments. Y.M. analyzed the data and wrote the article. G.Y. and H.D. revised the manuscript and contributed to discussions. B.X., Y.M. and Z.Q. contributed to discussions and important reagents and supported funds.

Yunhan Ma, and Baiyi Xie are Equally contributing.

Ethical Approval

This study was approved by the Animal Management Committee and the Animal Ethics Committee of Xiamen University.

Statement of Animal Rights

All procedures in this study were conducted in accordance with the Animal Management Committee and the Animal Ethics Committee of Xiamen University (XMULAC20170243) approved protocols.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: This work was supported by China Scholarship Council, the National Natural Science Foundation of China under Grant numbers 81771721, 81971505 and 81800664, the National Key Basic Research Program of China under Grant number 2018YFA0108304, the Natural Science Foundation of Fujian Province of China under Grant number 2016J01414, the Natural Science Foundation of Hunan Province of China (2019JJ50842) and Huxiang Young Talents of Hunan Province (2019RS2013).

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