Inhibition of interferon regulatory factor 4 orchestrates T cell dysfunction, extending mouse cardiac allograft survival

Wenjia Yuan[1], Hedong Zhang[1], Longkai Peng[1], Chao Chen[1,2], Chen Feng[1], Zhouqi Tang[1], Pengcheng Cui[2], Yaguang Li[1], Tengfang Li[1], Xia Qiu[2], Yan Cui[2], Yinqi Zeng[1], Jiadi Luo[3], Xubiao Xie[1], Yong Guo[1], Xin Jiang[4], Helong Dai[1,2]

[1]Department of Kidney Transplantation, Center of Organ Transplantation, The Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, China;
[2]Medical College, Guangxi University, Nanning, Guangxi 530004, China;
[3]Department of Pathology, The Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, China;
[4]Department of Organ Transplantation, The Fifth Clinical Medical College of Henan University of Chinese Medicine (Zhengzhou People's Hospital), Zhengzhou, Henan 450000, China.

Abstract

Background: T cell dysfunction, which includes exhaustion, anergy, and senescence, is a distinct T cell differentiation state that occurs after antigen exposure. Although T cell dysfunction has been a cornerstone of cancer immunotherapy, its potential in transplant research, while not yet as extensively explored, is attracting growing interest. Interferon regulatory factor 4 (IRF4) has been shown to play a pivotal role in inducing T cell dysfunction.

Methods: A novel ultra-low-dose combination of Trametinib and Rapamycin, targeting IRF4 inhibition, was employed to investigate T cell proliferation, apoptosis, cytokine secretion, expression of T-cell dysfunction-associated molecules, effects of mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) signaling pathways, and allograft survival in both *in vitro* and BALB/c to C57BL/6 mouse cardiac transplantation models.

Results: *In vitro*, blockade of IRF4 in T cells effectively inhibited T cell proliferation, increased apoptosis, and significantly upregulated the expression of programmed cell death protein 1 (PD-1), Helios, CD160, and cytotoxic T lymphocyte-associated antigen (CTLA-4), markers of T cell dysfunction. Furthermore, it suppressed the secretion of pro-inflammatory cytokines interferon (IFN)-γ and interleukin (IL)-17. Combining ultra-low-dose Trametinib (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) and Rapamycin (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) demonstrably extended graft survival, with 4 out of 5 mice exceeding 100 days post-transplantation. Moreover, analysis of grafts at day 7 confirmed sustained IFN regulatory factor 4 (IRF4) inhibition, enhanced PD-1 expression, and suppressed IFN-γ secretion, reinforcing the *in vivo* efficacy of this IRF4-targeting approach. The combination of Trametinib and Rapamycin synergistically inhibited the MAPK and mTOR signaling network, leading to a more pronounced suppression of IRF4 expression.

Conclusions: Targeting IRF4, a key regulator of T cell dysfunction, presents a promising avenue for inducing transplant immune tolerance. In this study, we demonstrate that a novel ultra-low-dose combination of Trametinib and Rapamycin synergistically suppresses the MAPK and mTOR signaling network, leading to profound IRF4 inhibition, promoting allograft acceptance, and offering a potential new therapeutic strategy for improved transplant outcomes. However, further research is necessary to elucidate the underlying pharmacological mechanisms and facilitate translation to clinical practice.

Keywords: Interferon regulatory factor 4; T cell dysfunction; Programmed death-1; Trametinib; Rapamycin; Mitogen-activated protein kinase; Mammalian target of rapamycin; Mouse cardiac transplantation

Introduction

T cells play a central role in acute allograft rejection, contributing to both T cell-mediated rejection and antibody-mediated rejection.[1,2] Following exposure to antigens, T cell dysfunction, which includes exhaustion, anergy, and senescence, is characterized by the impaired production of effector cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α), as well as an increase in the expression of inhibitory receptors, including programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG-3), CD160, CD244, and cytotoxic T lymphocyte-associated antigen (CTLA-4).[3,4]

The discovery of T cell dysfunction has been a major breakthrough in cancer immunology, paving the way for the development of innovative immunotherapies such as adoptive cell transfer and immune checkpoint inhibitors.[5,6] In contrast to reinvigorating dysfunctional T cells in cancer immunotherapy, transplant tolerance strategies focus on

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Wenjia Yuan and Hedong Zhang contributed equally to this work.

Correspondence to: Helong Dai, Department of Kidney Transplantation, Center of Organ Transplantation, The Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, China
E-Mail: helong68888@163.com

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deliberately inducing and maintaining a state of T cell dysfunction. An increase in the level of exhausted T cells is directly correlated with decreased allograft interstitial fibrosis and improved allograft function in clinical kidney transplant recipients.[7] The association between T cell dysfunction and superior postoperative outcomes suggests that promoting T cell dysfunction could be an effective strategy to improve the prognosis of organ transplantation.

IFN regulatory factor 4 (IRF4), a member of the interferon regulatory family, modulates immune responses through the IFN signaling pathway and plays a critical role in immune responses.[8] We have previously shown that depletion of IRF4 in $CD4^+$ T cell could induce donor-specific tolerance of mouse cardiac allografts by driving allogeneic T cell dysfunction, characterized by elevated expression of PD-1 and a decrease in the production of cytokines.[9,10] These results indicate that IRF4 controls the core regulatory circuit of T cell dysfunction; thus, the inhibition of IRF4 could serve as a potential therapeutic strategy to promote transplant acceptance.

Trametinib, a MEK1/2 inhibitor, exhibits dose-dependent inhibition of IRF4 expression. However, the efficacy of Trametinib alone is limited. *In vitro* studies demonstrated incomplete downregulation of IRF4 expression even at high concentrations of Trametinib (5 μmol/L).[10] In addition, 3 mg/kg Trametinib can only extend the median survival time of mouse cardiac allografts from approximately 7 days to 13 days, in contrast to transplant tolerance in IRF4 knock out (KO) mice.[10] IRF4 expression also relies on the activity of the mammalian target of Rapamycin (mTOR). Mechanistically, mTOR functions downstream of T cell receptor (TCR) stimulation signals to drive IRF4 expression and inhibition of the mTOR pathway downregulates IRF4 expression.[10,11]

Modern immunosuppressive regimens in transplantation rely on a combination drug approach, typically employing calcineurin inhibitor, antiproliferative agent, and corticosteroids, to achieve enhanced efficacy while minimizing drug-related toxicities.[12] Our study demonstrates that an ultra-low-dose combination of Trametinib and Rapamycin synergistically suppresses IRF4 expression. This collaborative treatment potently upregulates molecules linked to T cell dysfunction, such as PD-1, while simultaneously dampening the secretion of inflammatory cytokines by T cells. Remarkably, co-administration of Trametinib and Rapamycin at an extremely low dose (0.1 mg/kg each) induced immune tolerance in four out of five mice receiving cardiac transplants. These findings collectively suggest that synergistic IRF4 inhibition by Trametinib and Rapamycin holds immense promise as a therapeutic strategy for promoting allograft acceptance through the induction of T cell dysfunction.

Methods

Animals

Healthy male wildtype (WT) C57BL/6 and BALB/c mice were purchased from SLAC Experimental Animal Co., Ltd (Changsha, China). All mice were between 8 weeks of age and 12 weeks of age with body weights between 20 g and 25 g, and housed in an specific pathogen free environment. All animal experiments were performed at the Animal Experimental Center of the Second Xiangya Hospital of Central South University and adhered to the guidelines approved by the Animal Care Ethics Committee of Second Xiangya Hospital, People's Republic of China (No. 2020565).

Heterotopic heart transplantation model

Cardiac transplantation was performed as we previously described.[9,10] Fully vascularized hearts from donor BALB/c mice were heterotopically transplanted into the abdomen of recipient C57BL/6 mice. Graft survival was assessed daily by palpation of the recipient's abdomen. The unpalpable beating was considered cardiac graft rejection.

Cell culture

Whole spleen cells were obtained from C57BL/6 mice and T cells were isolated from whole spleen cells using the EasySep™ Mouse T Cell Isolation Kit (Stemcell, Vancouver, BC, Canada, 19851) according to the manufacturer's protocol. T cells were cultured in RPMI 1640 medium (Biochrome, Berlin, Germany) supplemented with 10% fetal calf serum (Linaris, Dossenheim, Germany), 10 mmol/L HEPES (MP Biomedicals, California, USA), 1 mmol/L sodium pyruvate (Sigma, Darmstadt, Germany), 1× non-essential amino acids (Sigma), and 10 mmol/L β-mercaptoethanol (Sigma). Splenocytes were plated at a density of 0.5×10^6 per well (96-well plate) and stimulated with interleukin-2 (IL-2, 1 ng/mL; Novoprotein, CK24), anti-CD28 (1 μg/mL; eBioscience, San Diego, USA, 102101), and anti-mouse CD3ε antibody (7.5 μg/mL, eBioscience). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was used to assess the *in vitro* proliferation of stimulated T cells.

Reagents and treatment protocol

Trametinib and Rapamycin with purities greater than 98% were purchased from Selleck Chemicals (Houston, TX, USA). *In vivo*, heterotopic heart transplant recipients were divided into four groups: dimethylsulfoxide (DMSO), mice were treated with the vehicle of Trametinib and Rapamycin; Trametinib (TRAM), mice were intraperitoneally injected with 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ Trametinib; Rapamycin (RAPA), mice were intraperitoneally injected with 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ Rapamycin; and Double used (DOU), mice were intraperitoneally injected with 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ of both Trametinib and Rapamycin. All treatments were administered from postoperative days (PODs) 0–15. Mice were euthanized upon graft rejection or upon reaching the predetermined endpoint, POD 100. *In vitro*, we cultured the cells into five groups: negative control (NC), cells were not treated with anti-CD3ε, anti-CD28, and IL-2; positive control (PC), cells were treated with anti-CD3ε (15 mg/mL, eBioscience, 14-0032-82), anti-CD28 (1 mg/mL, eBioscience, 102101), and IL-2

(20 ng/mL, Peprotech, Rocky Hill, NJ, USA), but no Trametinib and Rapamycin; Trametinib (TRAM), cells were treated with anti-CD3ε, anti-CD28, IL-2, and 100 nmol/L Trametinib; Rapamycin (RAPA), cells were treated with anti-CD3ε, anti-CD28, IL-2, and 100 nmol/L Rapamycin; and DOU, cells were treated with anti-CD3ε, anti-CD28, IL-2, 100 nmol/L Trametinib, and 100 nmol/L Rapamycin.

Flow cytometry

Heart, spleen, or lymph node cells were stained with specific surface or intracellular antibodies in the staining buffer. Cells were washed three times and analyzed on an FACS Canto II flow cytometer (BD Biosciences, USA) using FlowJo software (BD Biosciences). Cells were stained with anti-mouse CD3 (percp-cy5.5), anti-mouse CD4 (APC), anti-mouse CD8 (PE-cyanine7), anti-mouse CD44 (AF700), anti-mouse PD-1 (FITC), and anti-mouse IRF4 (PE) purchased from BioLegend (USA). Cells were labeled with CFSE using the Cell TraceTM CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA, USA).

ELISA

Serum levels of IFN-γ, IL-1β, IL-10, and IL-17 were measured using ELISA kits (Neobioscience, China, emc004.48) following the manufacturer's instructions. Samples were isolated at the time of collection, frozen on the same day, and stored at −80°C until further use. At specific times, nitrite absorption was measured using a Multiskan Sky-High spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 550 nm.

Reverse transcription-quantitative polymerase chain reaction

RNA was extracted from whole-cell lysates with a total RNA purification kit (CWBIO, Beijing, China, CW0580S) and then reverse-transcribed with a Revert Aid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA, K1622). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using a Quantstudio 5 Real-Time System (Thermo Fisher Scientific) with the ChamQ Universal SYBR qPCR Master Mix (Vazyme, China, Q711-02).

Western blot analysis

Protein was extracted using the radio immunoprecipitation assay buffer containing protease or phosphatase inhibitor cocktail (Antgene, China; Ant072). A bicinchoninic acid protein quantification kit (Vazyme, Nanjing, China; E112-02) was used to quantify the protein concentrations. Optical densities of bands of interest were determined using ImageJ 1.53c (National Institutes of Health, USA) and normalized against the appropriate loading controls.

Histology and immunofluorescence

Grafts were recovered at the end of the observation period. Samples were fixed in 7.5% formalin overnight and subsequently embedded in paraffin for 24 h. Paraffin blocks were sectioned and subjected to hematoxylin and eosin (H&E) staining. Slides were scanned using an Aperio ScanScope scanner (Aperio Technologies, Inc., Vista, CA, USA). Grading was performed following the International Society for Heart and Lung Transplantation (ISHLT) 2004 guidelines for cellular rejection scores. Immunofluorescence staining images were collected by an optical microscope (Nikon Eclipse E100, Tokyo, Japan), and the results were analyzed using ImageJ 1.53c (National Institutes of Health). Quantitative data with an image scale of 20 μmol/L ($n = 3$) and 10 visual fields were randomly selected, and the mean value was taken after manual counting.

RNA sequencing

Cells were collected in tubes containing 1 mL TRIzol reagent. RNA extraction, library preparation, and sequencing were performed at the BGI (Wuhan, China). DEGseq was used to identify differentially expressed genes (DEGs). Upregulated or downregulated genes were identified by Q <0.05 and fold change >2.0. Differential gene expression analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed by the Dr. Tom network platform of BGI (http://report.bgi.com/) for all cell differential genes.

Statistical analysis

All data are presented as the means ± standard deviation (SD). Two-tailed Student's *t*-test was used for comparisons between two groups. While differences among multiple groups were assessed using one-way analysis of variance (ANOVA). GraphPad software (version 8.0, USA) was used to analyze the data, and P values <0.05 were considered statistically significant.

Results

Trametinib and Rapamycin effectively reduces the expression of IRF4 in T cells, inducing T cell dysfunction in vitro

To determine the effects of this novel combination on T cell activation, $CD3^+$ T cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies, and subjected to treatment with Trametinib, Rapamycin, or a combination of the two. Cells were then cultured for three days at 37°C in a 5% CO_2 environment [Figure 1A]. Flow cytometry was used to quantify the expression levels of CD4, CD8, CD44, IRF4, and PD-1. Compared with either agent alone, the combination of Trametinib and Rapamycin significantly inhibited the expression of IRF4, reduced the proportion of $CD4^+CD44^+$ cells, and significantly increased the expression of PD-1. Although there was a significant increase in the expression of $CD4^+Foxp3^+$ in the DOU compared with the PC and the TRAM, there was no statistically significant difference between the DOU and the RAPA. Combined treatment with Trametinib and Rapamycin synergistically suppresses T cell activation, potentially promoting a regulatory T cell phenotype [Figure 1B].

$CD3^+$ T cells were stimulated, grouped, and cultured *in vitro* as described above to explore the effects of Trametinib and Rapamycin, administered alone or both,

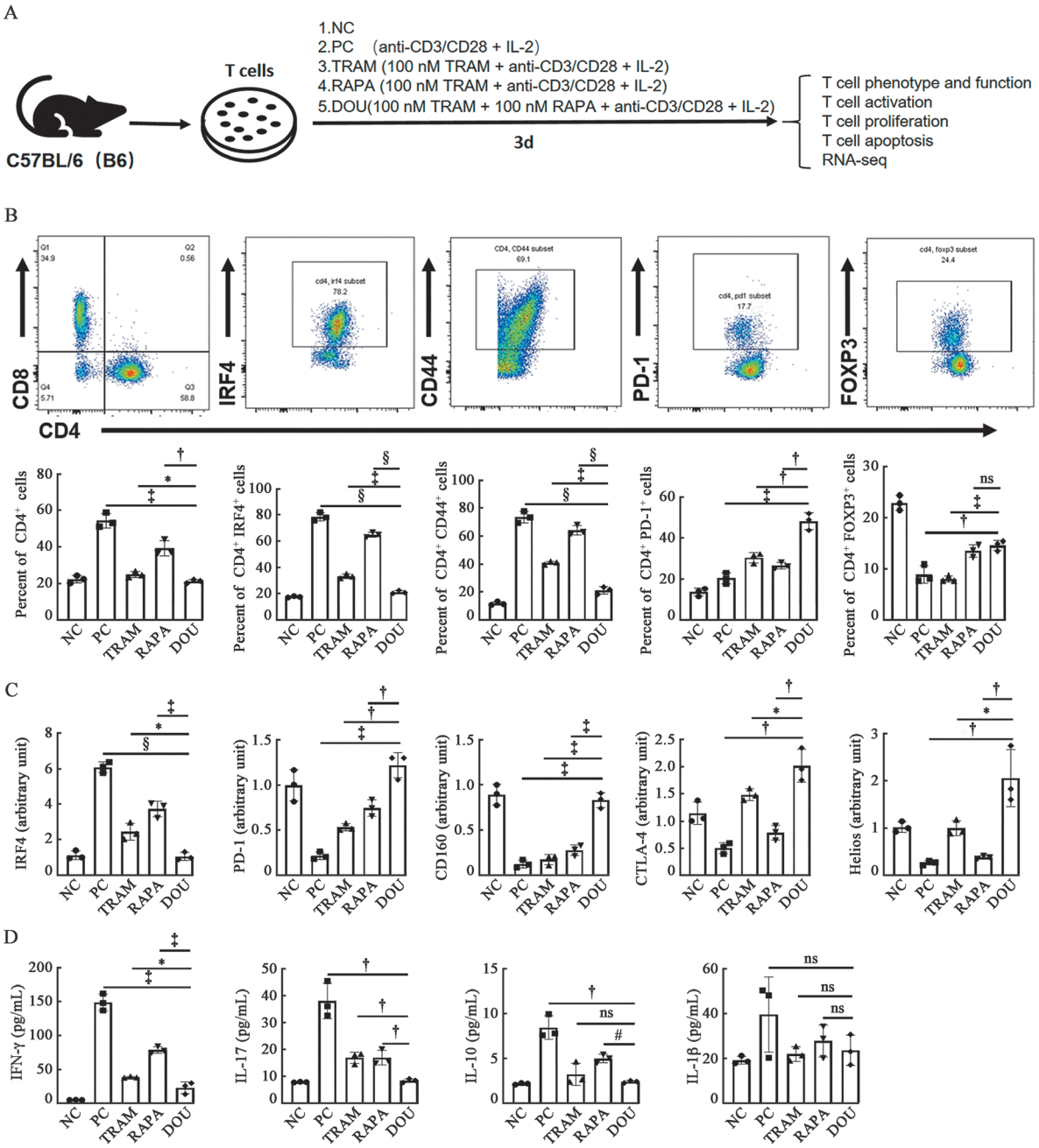

Figure 1: The specific effects of Trametinib and Rapamycin used alone or in combination on the T cell activation, cytokine secretion, expression of IRF4 in T cells, and T cell dysfunction markers *in vitro*. (A) CD3+ T cells were sorted from C57BL/6 mice spleen, stimulated with anti-CD3 and anti-CD28 mAbs, grouped according to the use of Trametinib and Rapamycin alone or in combination, and then cultured for three days in a 37°C and 5% CO_2 environment. (B) Representative FCM analysis of CD4+ T cells and the histogram graphs of the IRF4, CD44, PD-1, and Foxp3 on the gated CD4+ population of different groups are shown. (C) The total mRNA was extracted from T cells of all four groups, and the mRNA expressions of *IRF4*, *PD-1*, *Helios*, *CD160*, and *CTLA-4* were measured by qRT-PCR. (D) IFN-γ, IL-1β, IL-10, and IL-17 were measured by ELISA from the culture fluid of each group. $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$, $^{\S}P<0.0001$. CTLA-4: Cytotoxic T lymphocyte-associated antigen; DOU: Double used; FCM: Flow cytometry; IFN-γ: Interferon-gamma; IL: Interleukin; IRF4: Interferon regulatory factor 4; mAbs: Monoclonalantibodies; NC: Negative control; ns: Not significant; PC: Positive control; PD-1: Programmed cell death protein 1; qRT-PCR: Quantitative real-time polymerase chain reaction; RNA-seq: RNA sequencing.

on T cells cytokine secretion. The expression levels of *IRF4, PD-1, Helios, CD160*, and *CTLA-4* in each group were measured using qRT-PCR. The concentrations of cytokines, such as IFN-γ, IL-1β, IL-10, and IL-17, in the culture medium of each group were measured by ELISA. Compared with their individual use, the combined use of Trametinib and Rapamycin significantly reduced the expression of IRF4, increased the expression of PD-1, Helios, CD160, and CTLA-4 [Figure 1C], and reduced the secretion of IFN-γ and IL-17 [Figure 1D].

Trametinib and Rapamycin inhibit T cell proliferation and increase T cell apoptosis in vitro

To determine the effects of Trametinib and Rapamycin used alone or in combination on T cell proliferation and

apoptosis, CD3$^+$ T cells were purified, stimulated, grouped, and cultured *in vitro*, as described above. First, we used a CCK-8 cell proliferation assay to determine the specific effects of different concentrations of Trametinib and Rapamycin used individually or in combination on T cell proliferation [Figure 2A]. Results showed that treatment with either Trametinib or Rapamycin dose-dependently inhibited T cell proliferation, but their combination achieved better results. Similarly, results of the CFSE cell proliferation experiment confirmed that Trametinib and Rapamycin showed stronger inhibitory effects on T cell proliferation when used in combination than when they were administered individually [Figure 2B]. We also conducted Annexin V and 7AAD flow cytometry to examine the effects of Trametinib and Rapamycin, administered individually or in combination, on T cell apoptosis. Results revealed that the combined use of Trametinib and Rapamycin significantly promoted T cell apoptosis, particularly in the late-stage phase [Figure 2C].

mRNA-seq reveals the possible mechanisms of action of the combination of Trametinib and Rapamycin in T cells

Following the previously outlined methodology of stimulating, grouping, and *in vitro* culturing of CD3$^+$ T cells, the potential mechanisms underlying the combined impact of Trametinib and Rapamycin on T cells were explored. Following a culture period of three days, cells were collected from each group and subjected to mRNA transcriptome sequencing. Principal component analysis revealed a clear separation among the samples from the PC, TRAM, RAPA, and DOU groups, especially between the DOU and the other groups, despite the presence of some outliers in the NC group [Figure 3A]. DEGs identified using the transcriptome data of the DOU *vs*. PC, DOU *vs*. TRAM, and DOU *vs*. RAPA groups were analyzed. Comparative analysis showed that compared to the PC group, the DOU group had significantly more DEGs than the TRAM and RAPA groups. Comparisons to the NC group displayed similar patterns (Q <0.05, and fold change >2.0) [Figure 3B, C]. Finally, a KEGG pathway enrichment analysis was performed to determine the functional associations of the DEGs. Comparison of the DOU group with the PC, TRAM, and RAPA groups revealed DEGs involved in the top 20 pathways. Notably, significant differences in the Th1 and Th2 cell differentiation, Th17 cell differentiation, PD-L1 expression and PD-1 checkpoint pathway, and MAPK pathway were observed [Figure 3D]. This is consistent with our results that the combination of Trametinib and Rapamycin can affect the expression of PD-1,

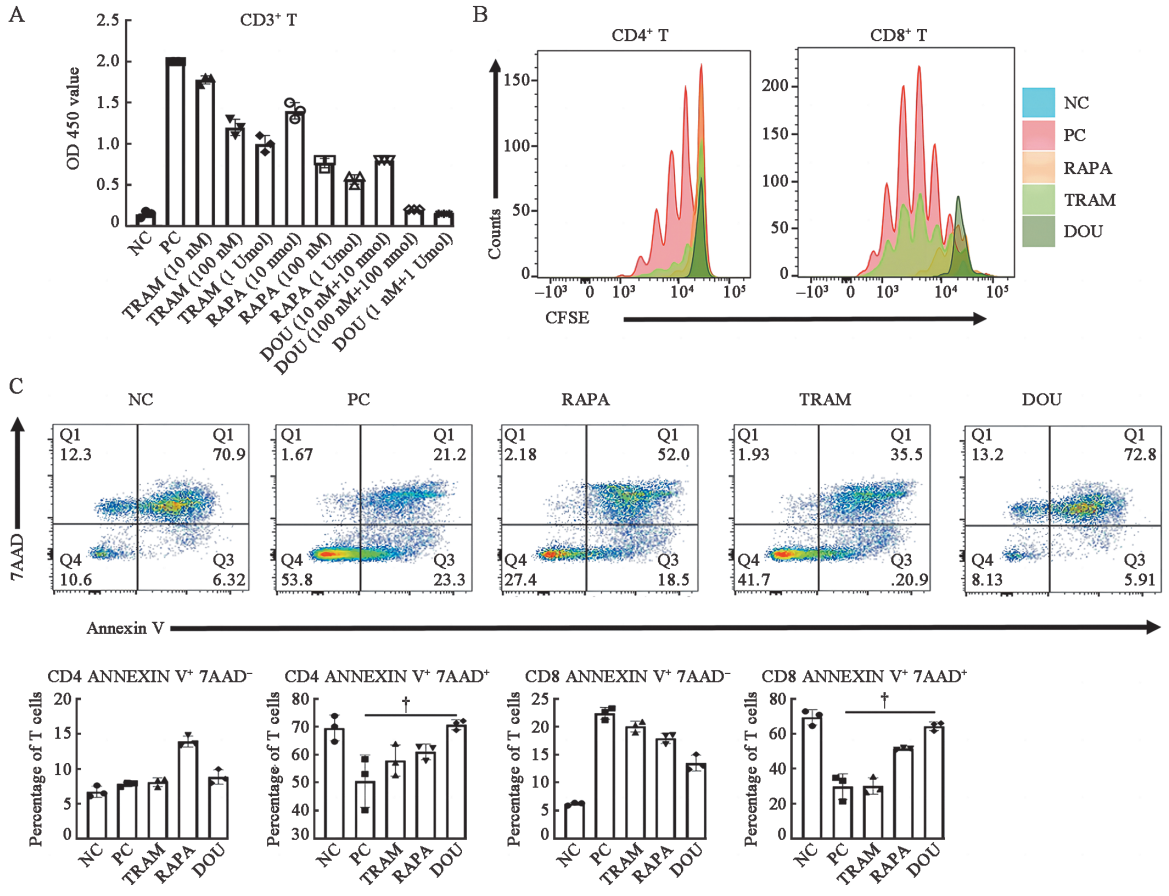

Figure 2: The effects of Trametinib and Rapamycin used alone or in combination on T cell proliferation and apoptosis *in vitro*. (A) The CCK-8 cell proliferation assay to explore the specific effects of different concentrations of Trametinib and Rapamycin used alone or in combination with CD3$^+$ T cells. OD 450 value of different groups are shown. (B) The analysis of CFSE-labeled CD4$^+$ T cells and CD8$^+$ T cells, compared with other groups, the formation of proliferative peak in DOU group was significantly inhibited. (C) Representative FCM analysis of T cell apoptosis (CD4$^+$ T cells and CD8$^+$ T cells) and the histogram graphs of the early-stage apoptosis (Annexin V$^+$ 7AAD$^-$), and late-stage apoptosis (Annexin V$^+$ 7AAD$^+$) of different groups are shown.$^\dagger P$ <0.01. CCK-8: Cell counting kit-8; CFSE: Carboxyfluorescein diacetate succinimidyl ester; DOU: Double used; FCM: Flow cytometry; NC: Negative control; OD 450: Optical density at 450 nm; PC: Positive control.

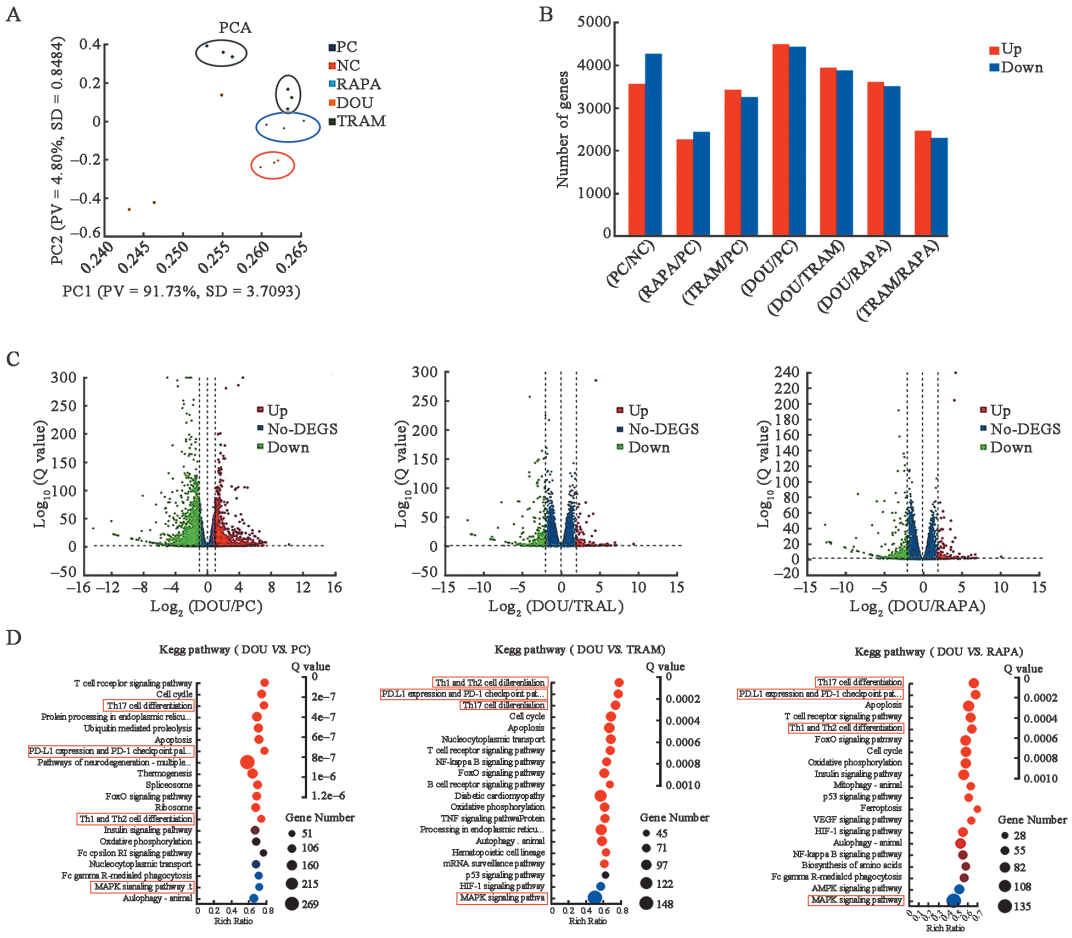

Figure 3: The possible mechanisms of action of the combination of Trametinib and Rapamycin in T cells. The T cells were sorted from C57BL/6 mice spleen, stimulated, cultured, and treated as before. The total mRNA was extracted from each group for RNA sequencing. (A) Principal component analysis revealed a clear separation between the samples from the PC, TRAM, RAPA, and DOU groups. (B) DEGs were analyzed in the transcriptome data of the DOU *vs.* PC, DOU *vs.* TRAM, and DOU *vs.* RAPA (Q <0.05, and fold change >2.0). (C) Volcano plot showing all up-regulated and down-regulated genes in DOU group compared with PC group, TRAM group and RAPA group. (D) KEGG pathway enrichment analysis of DEGs in DOU group compared with PC group, TRAM group, and RAPA group in accordance with biological process show DEGs involved in the top 20 pathways. The horizontal axis shows -Log_2 of the Q value. DEGs: Differentially expressed genes; DOU: Double used; PC: Positive control.

IFN-γ, and IL-17. At the same time, the results of KEGG suggest that MAPK pathway may play an important role in the combination of Trametinib and Rapamycin.

Ultra-low-dose combination of Trametinib and Rapamycin prolongs cardiac allograft survival and improves the pathological conditions

The effects of Trametinib and Rapamycin, administered individually or in combination, on allograft survival were investigated using a heterotopic heart transplantation model. Recipients received different treatments, and graft survival was monitored. In the DMSO group, heart grafts were acutely rejected (mean survival time [MST]:7.0 ± 0.8 days, n = 4), whereas recipients receiving Rapamycin treatment displayed prolonged graft survival time (MST: 19.2 ± 1.6 days, n = 5, P <0.01) compared with vehicle control. Similarly, the administration of Trametinib alone also prolonged graft survival time (MST: 15.2 ± 1.4 days, n = 5, P <0.01) compared with vehicle control. Recipients treated with a combination of Trametinib and Rapamycin exhibited the longest graft survival time (4 of 5 mice graft survival >100 days) compared to the untreated recipients or those receiving either Trametinib or Rapamycin alone (P <0.01) [Figure 4A, B]. Histopathological changes were assessed in the H&E-stained sections at day 7 post-transplantation. Compared with the other groups, the DOU group displayed significant reductions in tissue necrosis, diminished inflammatory cell infiltration, and lower ISHLT scores, indicating improved post-operative outcomes [Figure 4C, D].

Synergistic inhibition of MAPK and mTOR pathways by Trametinib and Rapamycin enhances IRF4 suppression and impairs T cell function

Much is already known about the MAPK and mTOR signaling pathway. To ascertain their potential role in our study, we investigated the effects of the combination of Trametinib and Rapamycin on MAPK and mTOR signaling pathways within $CD3^+$ T cells. T cells were purified, stimulated, grouped, and cultured *in vitro*, as described above [Figure 1A]. Western blotting was employed to detect the upstream and downstream

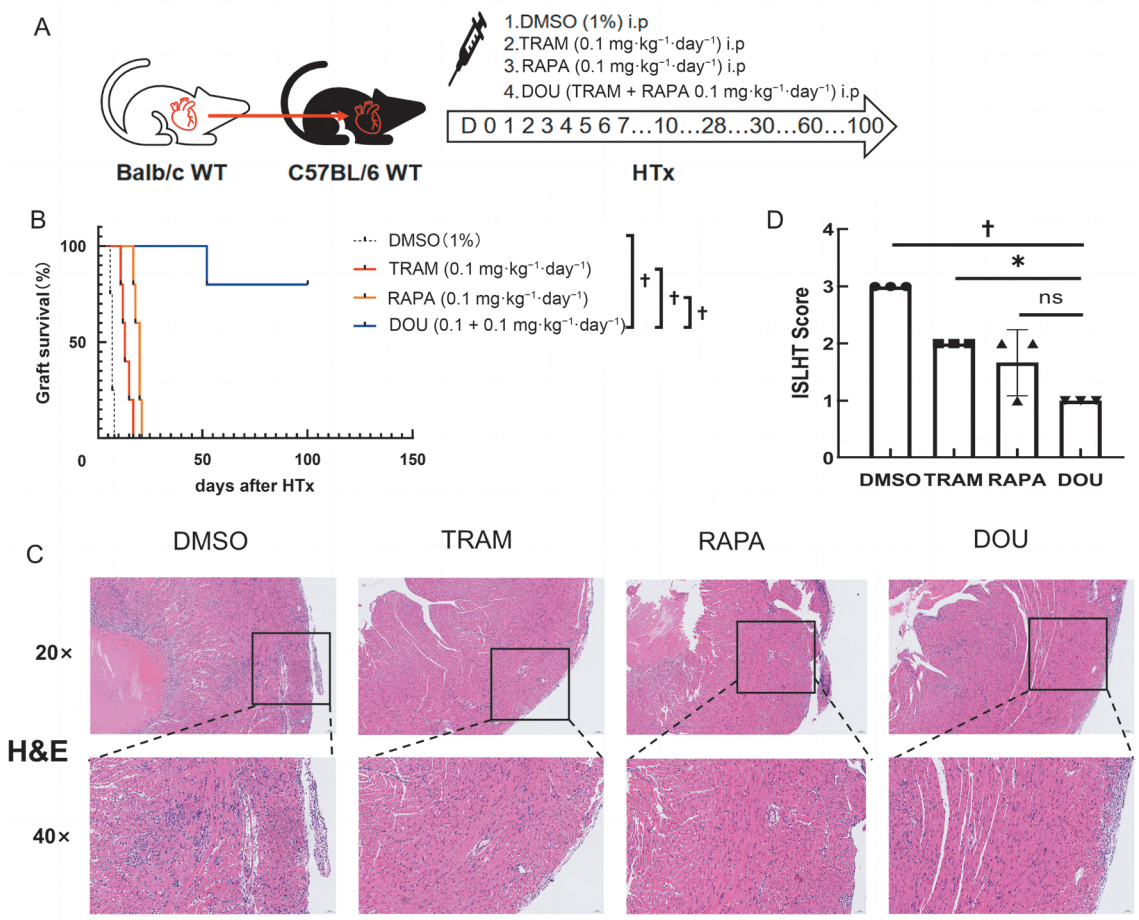

Figure 4: The effect of ultra-low-dose Trametinib and Rapamycin alone or in combination in a heterotopic heart transplantation model. (A) Hearts from donor BALB/c mice were heterotopically transplanted into the abdomen of recipient C57BL/6 mice and treated according to the group. (B) The statistical analysis of graft survival of Trametinib and Rapamycin alone or in combination and control with vehicle. †P <0.01. (C) H&E staining of cardiac grafts in recipients on POD7. Scale bars represent 100 μm and 50 μm. (D) According to the standard of ISHLT, evaluate the score of graft rejection among groups. *P <0.05, †P <0.01. DMSO: Dimethylsulfoxide; DOU: Double used; H&E: Hematoxylin and eosin; i.p.: Intraperitoneal; ns: Not significant; WT: Wildtype.

proteins of MAPK and mTOR Pathways [Figure 5A]. Consistent with its role as an MEK inhibitor, Trametinib significantly reduced the phosphorylation of MEK. Interestingly, the DOU group exhibited an even more pronounced inhibitory effect on phosphorylated MEK compared to Trametinib, and this difference was statistically significant. Similarly, the phosphorylated mTOR was significantly inhibited by Rapamycin as an inhibitor of mTOR, and the inhibitory effect was stronger in the DOU group. Encouragingly, a combination of Trametinib and Rapamycin demonstrates a more potent inhibitory capacity on some upstream and downstream proteins of MAPK and mTOR Pathways, such as p-ERK, p-RSK, p-AKT, Raptor, p-S6K, and p-4EBP1. Aligning with our previous results, the combination of Trametinib and Rapamycin exerted a more profound inhibitory effect on IRF4 expression, which leads to enhanced PD-1 expression [Figure 5A, B].

To examine the effect of the combined administration of ultra-low-dose Trametinib (0.1 mg·kg^{-1}·day^{-1}) and Rapamycin (0.1 mg·kg^{-1}·day^{-1}) both *in vivo*, we established a heterotopic heart transplant model. Our examination comprised various tissues and cell types (including grafts, lymph nodes, spleen, and serum) derived from recipients subjected to different treatments. RT-qPCR was conducted to detect the expression of *IRF4* in the transplanted hearts. Results showed that the expression of IRF4 in the DOU group was significantly lower than in other groups [Figure 5C].

In the spleen, we identified relevant immune cell populations, including B cells, NK cells, DCs, and macrophages. The proportions of these cells were comparable across groups; however, there is no significant difference among groups [Supplementary Figure 1, http://links.lww.com/CM9/C63]. In addition, there were no significant differences in the weight and cell number of the spleen [Supplementary Figure 2, http://links.lww.com/CM9/C63].

We used immunofluorescence to detect the expression levels of PD-1 in the grafts of each group. The expression of PD-1 was significantly higher in the DOU group than that in the other groups. These results are similar to those obtained *in vitro*, and PD-1, as a marker of T cell dysfunction, suggests that we might, as *in vitro*, extend the survival of the grafts by reducing IRF4 expression and inducing a T cell dysfunction phenotype through the

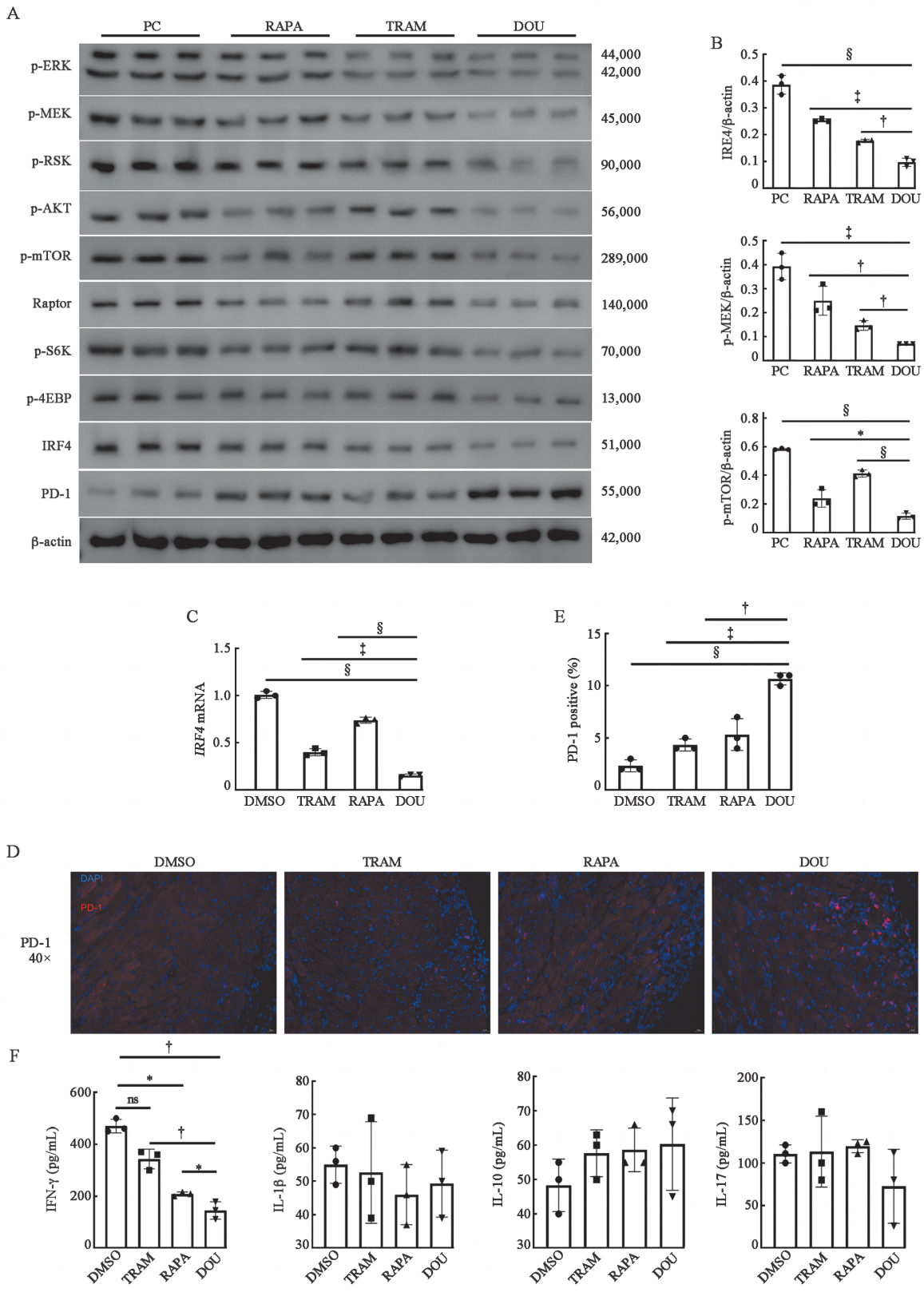

Figure 5: To ascertain the effects of the combination of Trametinib and Rapamycin on the MAPK and mTOR signaling pathways on CD3$^+$ T cells. (A) By western blotting, we analyzed the proteins with antibodies against phospho-ERK, phospho-MEK, phospho-RSK, phospho-AKT, Raptor, phospho-mTOR, phospho-S6K, phospho-4EBP1, IRF4, and PD-1. (B) Data are from one single experiment representative of three independent experiments with three biological replicates per group. Data were shown as means ± standard deviation of each group ($n = 3$); To determine whether the ultra-low-dose combined use of Trametinib and Rapamycin reduces the expression of IRF4 and induces a T cell dysfunction phenotype that affects graft survival. The mice heart transplantation model was established and grouped as before. (C) the mRNA expressions of IRF4 in the grafts were measured by qRT-PCR. (D) PD-1 in the grafts of each group was detected by immunofluorescence. (E) PD-1 percentage of immunofluorescence area, $n = 3$. (F) IFN-γ, IL-1β, IL-10, and IL-17 in serum were measured by ELISA. $^*P < 0.05$, $^\dagger P < 0.01$, $^\ddagger P < 0.001$, $^\S P < 0.0001$. DMSO: Dimethylsulfoxide; DOU: Double used; IFN-γ: Interferon-gamma; IL: Interleukin; IRF4: Interferon regulatory factor 4; mTOR: mammalian target of Rapamycin; ns: Not significant; PD-1: Programmed cell death protein 1.

ultra-low-dose combined use of Trametinib and Rapamycin [Figure 5D, E].

Finally, we measured the serum levels of cytokines, such as IFN-γ, IL-1β, IL-10, and IL-17, in recipients subjected to different treatments. Compared to the effect of the other treatments, the combined treatment reduced the serum concentration of IFN-γ, indirectly corroborating the observed changes in T cell function [Figure 5F].

Discussion

Research on T cell dysfunction in transplantation is relatively limited compared to that in cancer and infection. Fribourg *et al*[7] reported that pre-transplant lymphocyte-depleting induction therapy can remarkably increase circulating exhausted T cells and thus improve allograft function in kidney transplant recipients. In contrast, reinvigorating exhausted T cells by immune checkpoint blockade can result in allograft rejection while promoting tumor clearance.[11,13,14] This evidence from clinical organ transplantation highlights the promising prospect of improving transplant outcomes by promoting T cell dysfunction. Our study demonstrates that a novel, ultra-low-dose combination of Trametinib and Rapamycin synergistically inhibits IRF4 expression, promotes T cell dysfunction, and leads to a substantial prolongation of mouse cardiac allograft survival.

The reported intraperitoneal injection dose of Rapamycin in a mouse cardiac transplantation model ranges from 1.5 $mg \cdot kg^{-1} \cdot day^{-1}$ to 3 $mg \cdot kg^{-1} \cdot day^{-1}$,[15–18] whereas Trametinib is dosed at 1–3 $mg \cdot kg^{-1} \cdot day^{-1}$.[10,19–21] By contrast, notably 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ Trametinib and 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ Rapamycin were used in our study and showed a synergistic effect. Rapamycin can form a complex with FK506-binding protein (FKBP12) and inhibit mTOR complex 1 (mTORC1),[22] which can curb DNA and protein synthesis, leading to cell cycle arrest in late G1.[23] The MEK1/2 inhibitor Trametinib regulates cell proliferation, differentiation, survival, and motility by acting on the MAPK signaling pathway.[24] The mTOR and MAPK signaling pathways exhibit intricate crosstalk and compensatory mechanisms, forming a highly intertwined network that influences the pathogenesis of various diseases.[25] Dual inhibition of these pathways has emerged as a promising therapeutic strategy in cancer treatment.[26–28] Building on this concept, although Trametinib and Rapamycin have been documented as indirect inhibitors of IRF4, their individual use appears to result in incomplete inhibition.[10,29] Our western blot results also supported this concept. They demonstrate that the combination of Rapamycin and Trametinib exerts a more profound inhibitory effect on this signaling network compared to their individual application. This ultimately leads to enhanced IRF4 inhibition, suggesting potential convergence of both pathways in regulating IRF4 expression.

Due to the lack of a binding pocket, IRF4 is difficult to be developed into a direct small-molecule inhibitor. In addition to intervening in the mTOR and MAPK pathways, IRF4 antisense oligonucleotides can efficiently silence IRF4 expression in multiple myeloma and adult T cell leukemia lymphoma cell line.[30,31] After screening over 2000 compounds in multiple myeloma cell lines, a study found six drugs that can deplete IRF4 at 10 μmol/L. However, neither the names of the compounds nor the target receptor were disclosed.[32]

T cell dysfunction was first identified in chronic lymphocytic choriomeningitis virus infection.[33] Persistent antigen encounter and higher antigen load could be the causes of T cell dysfunction in the setting of chronic infection and cancer, which is similar to the case of transplantation with constant high-load alloantigen stimulation.[3,4] Promoting T cell exhaustion could be an effective strategy to improve transplant outcomes based on current evidence of better allograft function and T cell dysfunction.[7,34] In organ transplantation, multiple studies have shown that a high antigen load or antigen load to T cell abundance ratio is the main driver of transplant tolerance and T cell dysfunction.[35–38] Lymphocyte-depleting induction therapy enriches circulating exhausted T cells and correlates with better allograft function, which also might be a result of an increased antigen load-to-T cell abundance ratio.[7] However, the current cornerstone of transplantation immunosuppression, calcineurin inhibitors, impairs the induction of the HMG-box transcription factor TOX, which is a central regulator governing T cell dysfunction.[39] Therefore, novel immunosuppressants are needed to improve long-term transplant outcomes by promoting T cell dysfunction. With this ultra-low-dose combination of Trametinib and Rapamycin, four of five mice achieved long-term graft survival in a mouse cardiac transplant model by promoting T cell dysfunction.

The impact of Rapamycin on T cell dysfunction remains a controversial topic. Our findings align with those of a previous study that indicated that Rapamycin upregulates PD-1 expression in kidney transplant recipients, countering the effect of tacrolimus.[40] However, a different study showed that mTOR signaling drives the expression of T cell dysfunction markers in response to cytokines, a process mitigated by mTOR inhibition.[41] This discrepancy might be attributed to differences in immune settings.

Under treatment with combination of Trametinib and rapamycin, KEGG pathway enrichment analysis of our mRNA sequencing data revealed significant alterations in pathways associated with T cell differentiation and PD-L1/PD-1 signaling, suggesting complex immune regulation. While this study demonstrates the promise of Trametinib and Rapamycin in inhibiting IRF4, inducing T cell dysfunction and extending graft survival, certain limitations warrant further investigation. Most importantly, the precise molecular and cellular mechanisms underlying the synergistic action of these drugs on IRF4 expression inhibition require detailed exploration and verification. Furthermore, it is unclear whether checkpoint blockade can abrogate transplant tolerance established through this novel Trametinib- and Rapamycin-combination-induced T cell dysfunction.

In conclusion, our study demonstrates the synergistic potential of a novel, ultra-low-dose combination of

Trametinib and Rapamycin synergistically suppresses the MAPK and mTOR signaling network, leading to profound IRF4 inhibition, and inducing T cell dysfunction. This unique strategy significantly prolonged graft survival in our mouse cardiac transplant model, highlighting its potential to improve transplant outcomes. While further research is crucial to elucidate underlying molecular and cellular mechanisms and translate them into clinical practice, our work offers a promising step towards improved long-term graft survival via manipulating T cell dysfunction.

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Conflicts of interest

None.

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