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Research article

Oxymatrine combined with rapamycin to attenuate acute cardiac allograft rejection

Xu Lan [a,1], Jingyi Zhang [b,c,d,1], Shaohua Ren [b,c,d,1], Hongda Wang [b,c,d], Bo Shao [b,c,d], Yafei Qin [b,c,d], Hong Qin [b,c,d], Chenglu Sun [b,c,d], Yanglin Zhu [b,c,d], Guangming Li [b,c,d], Hao Wang [b,c,d,*]

[a] *Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing, 100029, China*
[b] *Department of General Surgery, Tianjin Medical University General Hospital, Tianjin, 300052, China*
[c] *Tianjin General Surgery Institute, Tianjin Medical University General Hospital, Tianjin, 300052, China*
[d] *Tianjin Key Laboratory of Precise Vascular Reconstruction and Organ Function Repair, Tianjin 300052, China*

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ABSTRACT

Background and aim: Solid organ transplantation remains a life-saving therapeutic option for patients with end-stage organ dysfunction. Acute cellular rejection (ACR), dominated by dendritic cells (DCs) and $CD4^+$ T cells, is a major cause of post-transplant mortality. Inhibiting DC maturation and directing the differentiation of $CD4^+$ T cells toward immunosuppression are keys to inhibiting ACR. We propose that oxymatrine (OMT), a quinolizidine alkaloid, either alone or in combination with rapamycin (RAPA), attenuates ACR by inhibiting the mTOR–HIF–1α pathway.
Methods: Graft damage was assessed using haematoxylin and eosin staining. Intragraft $CD11c^+$ and $CD4^+$ cell infiltrations were detected using immunohistochemical staining. The proportions of mature DCs, T helper (Th) 1, Th17, and Treg cells in the spleen; donor-specific antibody (DSA) secretion in the serum; mTOR–HIF–1α expression in the grafts; and $CD4^+$ cells and bone marrow-derived DCs (BMDCs) were evaluated using flow cytometry.
Results: OMT, either alone or in combination with RAPA, significantly alleviated pathological damage; decreased $CD4^+$ and $CD11c^+$ cell infiltration in cardiac allografts; reduced the proportion of mature DCs, Th1 and Th17 cells; increased the proportion of Tregs in recipient spleens; downregulated DSA production; and inhibited mTOR and HIF-1α expression in the grafts. OMT suppresses mTOR and HIF-1α expression in BMDCs and $CD4^+$ T cells *in vitro*.

Abbreviations: ACR, acute cellular rejection; APC, antigen-presenting cell; BMDCs, bone marrow-derived dendritic cells; DC, dendritic cell; DSA, donor-specific antibody; enzyme-linked immunosorbent assay, ELISA; H&E, haematoxylin and eosin; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MHC class II, major histocompatibility complex class II; MST, median survival time; OMT, oxymatrine; p, P value; POD, postoperative day; Th17, T helper 17 cell; TNF, tumor necrosis factor; Treg, regulatory T cell.

* Corresponding author. Department of General Surgery, Tianjin Medical University General Hospital, 154 Anshan Road, Heping District, Tianjin, 300052, China.,

E-mail addresses: lanxu987@126.com (X. Lan), 815509607@qq.com (J. Zhang), shaohua.r@foxmail.com (S. Ren), wanghd0326@163.com (H. Wang), 15552849287@163.com (B. Shao), qinyafei92@tmu.edu.cn (Y. Qin), hongqin.hust@foxmail.com (H. Qin), scl17664135084@163.com (C. Sun), zalljwy@163.com (Y. Zhu), liguang1118@163.com (G. Li), hwang1@tmu.edu.cn (H. Wang).

[1] Xu Lan, Jingyi Zhang, and Shaohua Ren contributed equally to this work.

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Conclusions: Our study suggests that OMT-based therapy can significantly attenuate acute cardiac allograft rejection by inhibiting DC maturation and $CD4^+$ T cell responses. This process may be related to the inhibition of the mTOR–HIF–1α signaling pathway by OMT.

1. Introduction

Solid organ transplantation remains a treatment option that can save the lives of patients with organ dysfunction. Acute cellular rejection (ACR) is a major causes of death following transplantation [1,2]. It is predominantly caused by T cells and is accompanied by myocardial damage and the infiltration of inflammatory cells. The fundamental mechanics of the ACR have attracted the interest of researchers. Dendritic cells (DCs) derived from the bone marrow are dominant antigen-presenting cells (APCs) that trigger both innate and adaptive immunity via T cells in response to signals or antigens [3]. In cases of acute cardiac allograft rejection, $CD4^+$ T cells play a crucial role as effector cells, differentiating into T helper (Th) and Th17 cells when stimulated by antigens presented by DCs [3–6]. Direct allorecognition by host T cells has been the principal focus of research on the manipulation of cell interactions. Direct allorecognition occurs when host T cells detect intact non-self-major histocompatibility complex (MHC) molecules on the surfaces of foreign DCs that differ from the host. It is anticipated to exert a dominant influence on early immunological responses after allografting [7]. Besides cellular immunity, humoral immunity plays an indispensable role in graft rejection mainly through antibody secretion [8]. DCs play a crucial role in the initiation and regulation of antibody synthesis through direct interaction with B cells [9]. In addition, IgG enhances the immune responses of $CD4^+$ T cells to protein antigens by activating Fcγ-receptors [10]. The occurrence of both acute and chronic graft rejection is significantly reduced with the use of antibodies, immunoglobulins, and other immunosuppressive medications. However, the use of immunosuppressants is limited by severe adverse effects including nephrotoxicity with calcitonin inhibitors and poor wound healing, which can be attributed to mammalian target of rapamycin (RAPA) inhibitors [11]. Therefore, researchers are currently investigating the potential of novel immunosuppressive drugs.

Some herbs or extracts, such as *Sophora flavescens*, have anti-inflammatory or immunomodulatory effects [12,13]. In traditional Chinese medicine and modern research, *S. flavescens* exerts anti-inflammatory and antiviral effects [14]. Oxymatrine (OMT) is a quinolizidine alkaloid derived from *S. flavescens* that possesses excellent physicochemical stability [15]. The molecular structure of OMT was $C_{15}H_{24}N_2O_2$ and its molecular weight was calculated to be 264 g/mol. A variety of studies have demonstrated that OMT has many therapeutic effects, such as organ- and tissue-protection, antitumor, and antiviral effects, in different disease models [16]. OMT has a substantial effect on Dextran Sulfate Sodium (DSS) -induced acute intestinal inflammation due to its anti-inflammatory properties [13,17,18]. Furthermore, OMT has the potential to enhance the production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor (TGF)-β, while reducing the production of pro-inflammatory cytokines such as IL-6, IL-17A, and interferon (IFN)-γ [18–23].

Mammalian target of RAPA (mTOR), a serine-threonine kinase, is evolutionarily conserved in mammals. Inhibiting mTOR leads to immunosuppressive effects [24,25]. Both *in vivo* and *in vitro* studies have demonstrated that mTOR inhibition inhibits DC maturation [26]. Moreover, Th1 and Th17 differentiation require the presence of mTOR, whereas mTOR deficiency promotes Treg differentiation [27]. In addition, the connection between hypoxic signals and inflammation is especially close. Activation of the inflammatory pathway and alteration of the immune cell phenotype and function can be exacerbated by hypoxia, leading to the aggravation of the inflammatory response. Simultaneously, oxygen consumption by inflammatory reactions can aggravate organ hypoxia [28]. The protein known as hypoxia-inducible factor (HIF)-1α is a crucial component that cells produce as a reaction to hypoxia. HIF-1α has positive effects on migration and proinflammatory function in DCs [29]. HIF-1α can bind to Foxp3 and induce its degradation, thereby inhibiting Treg differentiation [30]. OMT may suppress the expression of both mTOR and HIF-1α in tumor cells to achieve anti-tumor effects [30–32].

RAPA is a macrolide immunosuppressant that inhibits the cell cycle in the G1 phase and can inhibit cell- and antibody-mediated rejection through immunosuppressive effects [33]. mTOR inhibitors (mTOR-Is), such as RAPA, impair DC endocytic functions and reduce MHC-II expression. mTOR-I also decreased T cell stimulation and proliferation induced by DCs, as well as lower IFN-γ and IL-17 protein and mRNA levels in spleen cells. mTOR-I inhibits $CD4^+$ T helper cells and may be beneficial for Treg induction The inhibition of mTOR increases the number of Tregs, regardless of the antigen level. Moreover, mTOR-I enhanced Foxp3 expression in a transforming growth factor-β (TGF-β)-dependent manner. Clinical transplantation studies have indicated that the inclusion of mTOR-Is in immunosuppressive therapy leads to lower levels of new DSAs, suggesting that maintaining adequate mTOR-I concentrations could help prevent antibody-mediated rejection [33]. The development of novel immunosuppressive drugs without serious side effects as adjuvant compounds in combination with RAPA, to enhance their protective effects, remains a focus of investigation.

2. Materials and methods

2.1. Animals

Male C57BL/6 and BALB/c mice, with a weight range of 22–25 g and an age range of 6–8 weeks, were procured from the China Food and Drug Inspection Institute (Beijing, China). The animal experiments and studies adhered to the regulations established by the Chinese Association for the Protection of Animals and were conducted in compliance with the protocols sanctioned by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China, Ethic No. IRB2022-DW-23). The animals were housed in a

thermostatic chamber at the Tianjin Institute of General Surgery and provided with unrestricted access to food and water.

2.2. Heterotopic cardiac transplantation and experimental groups

Intra-abdominal heterotopic cardiac transplantation was performed by transplanting donor hearts from BALB/c mice into the abdomens of C57BL/6 mice. The survival of the graft was determined on a daily basis by palpating the abdomen of the recipient. The unpalpable beating was regarded as a cardiac graft rejection and was proven visually after laparotomy. OMT was dissolved in 0.9% saline and administered intraperitoneally to C57BL/6 recipient mice. RAPA was dissolved in 100% olive oil and administered subcutaneously to recipient C57BL/6 mice (2 mg/kg/d). 0.9% physiological saline was used for vehicle control. The recipient C57BL/6 mice were assigned randomly to four groups (n = 6 for each group): Group 1, untreated; Group 2, OMT treated (100 mg/kg/d); Group 3, RAPA treated (2 mg/kg/d); and Group 4, OMT (100 mg/kg/d) + RAPA (2 mg/kg/d) treated. Recipients were given either OMT or RAPA once per day from the day of the transplantation (POD 0) to POD 8. The dosages and administration of OMT and RAPA were determined according to the high dose group and RAPA-treated group of the previous experiment [34]. In addition, 4 more transplants for each group were performed to evaluate graft survival only (n = 10 for each group). The schematic diagram of the experimental design was shown in the graphical abstract.

2.3. H&E staining

Specimens of cardiac grafts were obtained on POD 8 and fixed in 10% formalin. The specimens were subjected to haematoxylin and eosin (H&E) staining using 5 μm paraffin-embedded slices. Rejection severity was evaluated using a light microscope. Graft rejection included lymphocytic infiltration, vasculitis, thrombosis, myocardial necrosis, and hemorrhage, and graft specimens were scored according to the International Society for Heart and Lung Transplantation (ISHLT), which has established a standard grading system for acute cellular rejection in cardiac biopsies. This system includes four grades: grade 0, indicating no rejection; grade 1, characterized by interstitial and/or perivascular infiltrate with up to one focus of myocyte damage; grade 2, denoting two or more foci of infiltrate with associated myocyte damage; and grade 3, indicating diffuse infiltrate with multifocal myocyte damage, edema, hemorrhage, and vasculitis [35].

2.4. Immunohistochemistry staining

Immunohistochemistry with biotin-conjugated rat anti-mouse $CD4^+$ and $CD11c^+$ monoclonal antibodies (Abcam, Shanghai, China, http://www. abcam. cn) was used to analyze intragraft $CD4^+$ and $CD11c^+$ cell infiltrations. Non-specific staining was evaluated using negative control sections omitting the primary antibodies. To quantify the number of infiltrating $CD4^+$ and $CD11c^+$ cells within the graft, all positively stained cells within a given section were counted (cells/mm^2). Utilizing ImageJ software (National Institutes of Health, Bethesda, Maryland, United States), quantification was performed.

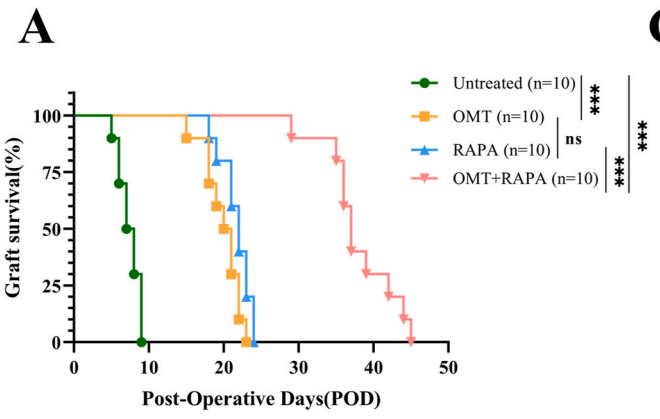
2.5. Flow cytometry analysis

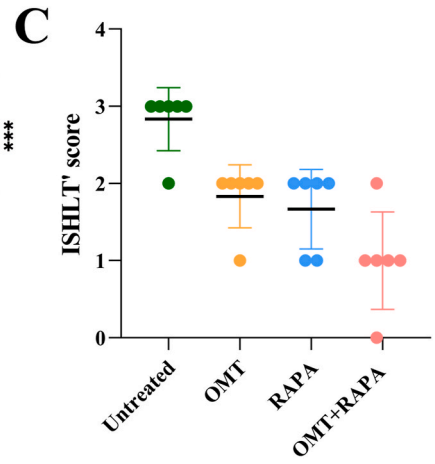
The mouse spleens were collected from different groups after sacrifice. The obtained splenocytes were resuspended with 2 ml of PBS and 100 μl of splenocyte suspension was added to each flow tube. The technique of flow cytometry was employed to discern distinct populations of immune cells. The investigation utilized monoclonal antibodies and reagents from BioLegend or eBioscience, including Zombie NIR™ Dye (Dead/Live reagent, 0.2 μl/tube), CD11c (PerCP-labelled, 1 μl/tube), CD80 (PE-labelled, 1 μl/tube), MHC class II (APC-labelled, 0.5 μl/tube), anti-mouse CD4 (FITC-labelled, 0.5 μl/tube), IFN-γ (PE-labelled, 1 μl/tube), IL-17 (Percp-labelled, 1 μl/tube), CD25 (PE-labelled, 1 μl/tube), Foxp3 (APC-labelled, 1 μl/tube), p-mTOR (PE-labelled, 2.5 μl/tube), HIF-1α (APC-labelled, 2.5 μl/tube) to detect DCs ($CD11c^+$, $CD80^+$, MHC class II^+), Th1 cells ($CD4^+$ and IFN-γ^+), Th17 cells ($CD4^+$ and IL-$17\alpha^+$), Tregs ($CD4^+$, $CD25^+$ and $Foxp3^+$), and mTOR–HIF–1α signaling pathway in $CD11c^+$ cells and $CD4^+$ cells in both spleens and allografts. In addition, the splenocytes were subjected to an initial coincubation with stimulators for a duration of 6 h, after which fluorescent antibody staining was performed to accurately detect the presence of Th1 and Th17 cells.

Furthermore, on POD8, 5 μl of serum was taken from C57BL/6 recipients to determine the fraction of donor-specific antibody (DSA). Donor BALB/c splenocytes (5×10^5 cells) were co-cultured with serum diluted at 1:10 in PBS for 30 min at 37°C, and then stained with anti-CD3 (FITC-labelled, 1 μl/tube) and anti-IgG (PE-labelled, 0.5 μl/tube) antibody (for IgG measurement) or anti-CD3 (FITC-labelled) and anti-IgM (PE-labelled, 0.5 μl/tube) antibody (for IgM measurement). Considering the potential for donor-specific antibodies to interact through their Fc segments with Fc receptor-owing cells, this method relies on CD3 T cells isolated from the donor to assess the proportion of alloreactive antibodies.

2.6. Isolation of mononuclear cells from the grafts

On POD 8, the transplanted grafts were retrieved and deposited in heparinized saline (HS). Due to the limited size of the grafts, mononuclear cells were extracted from a portion of each graft. Following the excision of the epicardial adipose tissue, the cardiac grafts underwent fragmentation and subsequent irrigation with HS to remove any residual blood. The minced tissue was digested for 90 min at 37 °C with type II collagenase (2 mg/mL; Solarbio, Beijing, China), DNase I (100 μg/mL; Solarbio, Beijing, China), and trypsin (0.1%; Solarbio, Beijing, China) in 5 mL of RPMI 1640 medium (Hyclone, USA). Subsequently, 5 mL of a serum-containing medium





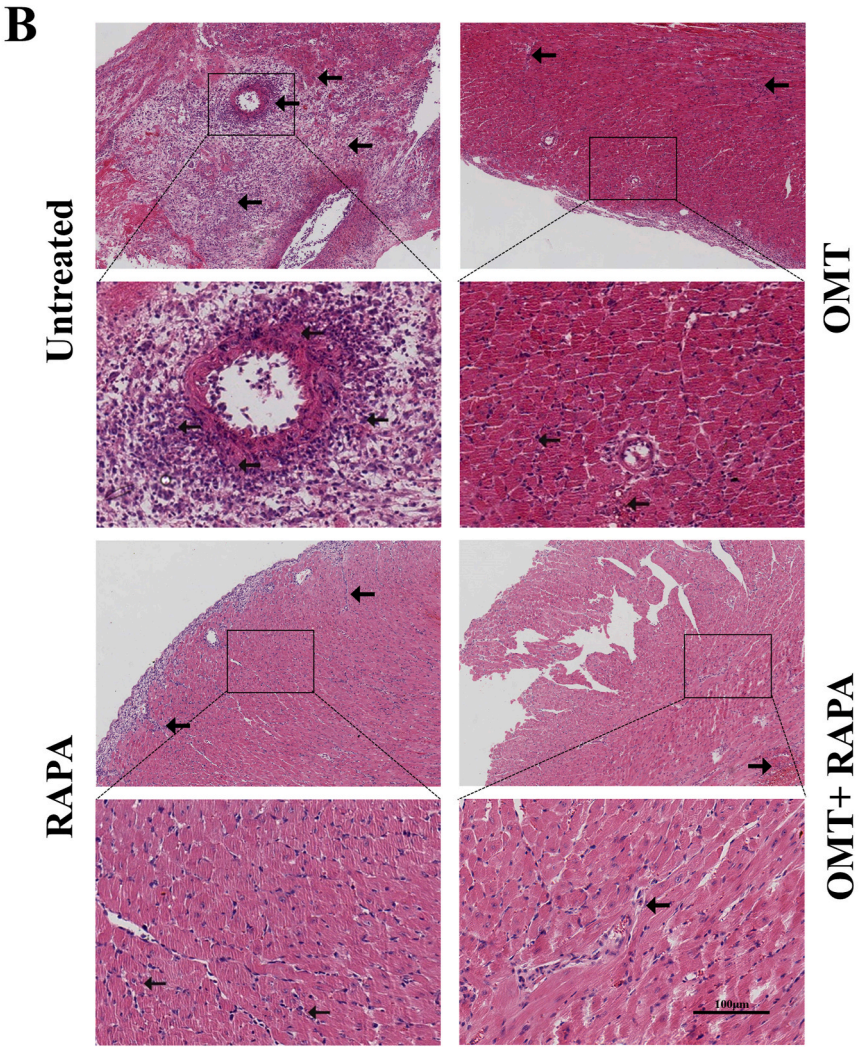


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Fig. 1. OMT alleviated pathological damage on cardiac allografts. (A) Survival time of cardiac allografts in each group (n = 10 for each group). (B) The cardiac allografts from each group were collected at postoperative day 8 and stained with H&E. The arrows indicate vasculitis, inflammatory cell infiltration, myocyte necrosis, and structural disorganization of myocytes in the untreated group, and myocyte damage in OMT treated group and RAPA treated group (400 μm). The arrows indicate lymphocytes diffuse infiltrate with multifocal myocyte damage and vasculitis in the untreated group, and distorted the myocyte architecture in OMT treated group, RAPA treated group, and the combination group (100 μm). (C) According to the standard of ISHLT, the score of graft rejection was evaluated among different groups. ISHLT score statistical analysis was done by one-way ANOVA followed by the LSD test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Values are shown as the mean ± SEM.

was put into terminate the process of digestion. The cells were filtered using a 70 μm cell strainer and centrifuged at 300 g for 5 min at 4 °C. Removed the supernatant, and then meticulously resuspended the cell pellet in 40% Percoll and 2 mL of 70% Percoll, about 3 mL. After centrifugation, the mononuclear cells in the intermediate white layer were collected for flow cytometry analysis.

2.7. Isolate and culture $CD4^+$ T cells

Using the CD4 microbeads kit (Precision Biomedicals, Tianjin, China), we were able to isolate naive $CD4^+$ T cells from splenocytes of C57BL/6 mice. Anti-mouse CD3 (100 ng/ml), CD28 (200 ng/ml) antibodies, and IL-2 (ebioscience Inc. USA) were used to stimulate $CD4^+$ T cell activation. Isolated $CD4^+$ T cells (1×10^6) were then used in subsequent experiments in 96-well plates.

2.8. Isolate and culture bone marrow-derived DCs and the administration of OMT

From the femur and tibia of C57BL/6 mice, bone marrow-derived dendritic cells (BMDCs) were flushed. BMDCs lysed with red blood cells were cultured in 48-well culture plates (Greiner Bio-One Japan, Tokyo, Japan) with complete medium (PeproTech, Cranbury, NJ) containing GM-CSF (20 ng/ml). On day 3, adjusting the media to achieve a final concentration of 10 ng/ml of GM-CSF for the entire culture. On day 6, discarding the extant medium from the plate and introducing a fresh medium to achieve a total culture concentration of 10 ng/ml of GM-CSF and 10 ng/ml of IL-4. On day 8, discarding the extant medium from the plate and substituting it with a fresh medium to achieve a final concentration of 5 ng/ml of GM-CSF and 10 ng/ml of IL-4 in the entire culture. On day 10, BMDC culture was complete, and the stimulants and drugs were added. To induce maturation, BMDCs were stimulated with 100 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich) for a duration of 12 h. In the presence of LPS, the medium was supplemented with a variety of reagents. On day 11, we harvested BMDCs from different groups for subsequent experiments [36].

2.9. Statistics

The statistical analyses were performed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA), and the results were presented using GraphPad Prism 8 software (GraphPad Prism Software Inc., San Diego, CA). Graphical abstract created with BioRender.com. The data were presented as mean ± standard error of mean (SEM). The median (P0.25, P0.75) cardiac graft survival time was evaluated using the Kaplan-Meier cumulative survival method, and the Log-rank (Mantel-Cox) test was employed to compare survival differences among groups. One-way analysis of variance (ANOVA) was used to analyze the differences among multiple groups. The least significant difference (LSD) test was used for post hoc analysis of datasets composed. A P value (p) < 0.05 was considered statistically significant ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

3. Results

3.1. OMT either alone or in combination with RAPA relieved pathological damages of cardiac allografts

In order to assess the viability of OMT as a means of mitigating acute allograft rejection, we conducted a heterotopic heart transplantation procedure utilizing BALB/c donor mice and C57BL/6 recipient mice. Our objective was to investigate whether OMT could extend graft survival. As shown in Fig. 1A, OMT resulted in a significant increase in the median survival time (MST) of cardiac allografts (MST: 19.5 days) when compared to the untreated group (MST: 7.5 days) ($p < 0.001$). Compared with the RAPA-treated group (MST: 22 days), the graft survival time of the combination therapy group was further prolonged to MST of 37 days ($p < 0.001$)

The transplanted cardiac allografts were collected on POD 8 for H&E staining to assess pathological damage. Obviously, histology showed significant inflammatory cell infiltration around the coronary arteries, myocytenecrosis, and structural disorganization of myocytes in the untreated group. In contrast, the grafts in groups treated with either OMT, RAPA, or OMT + RAPA showed a significant alleviation of pathological damage. The OMT-treated group and the RAPA-treated group showed only a few areas of myocyte damage and architectural distortion on histology. Furthermore, the OMT + RAPA treated group showed normal histology on POD8 (Fig. 1B). Subsequently, according to the ISHLT grading system, grafts in the untreated group exhibited high levels of inflammatory cell infiltration and severe myocyte injury (scored 3), the damages to the grafts of OMT or RAPA monotherapy group was scored 2, and the pathology damage severity of the grafts in OMT + RAPA combination therapy group was scored 1 (Fig. 1C). These results suggest that OMT has a protective effect against acute allograft rejection, and combination therapy with OMT and RAPA further prolonged graft survival compared with that of monotherapy group.

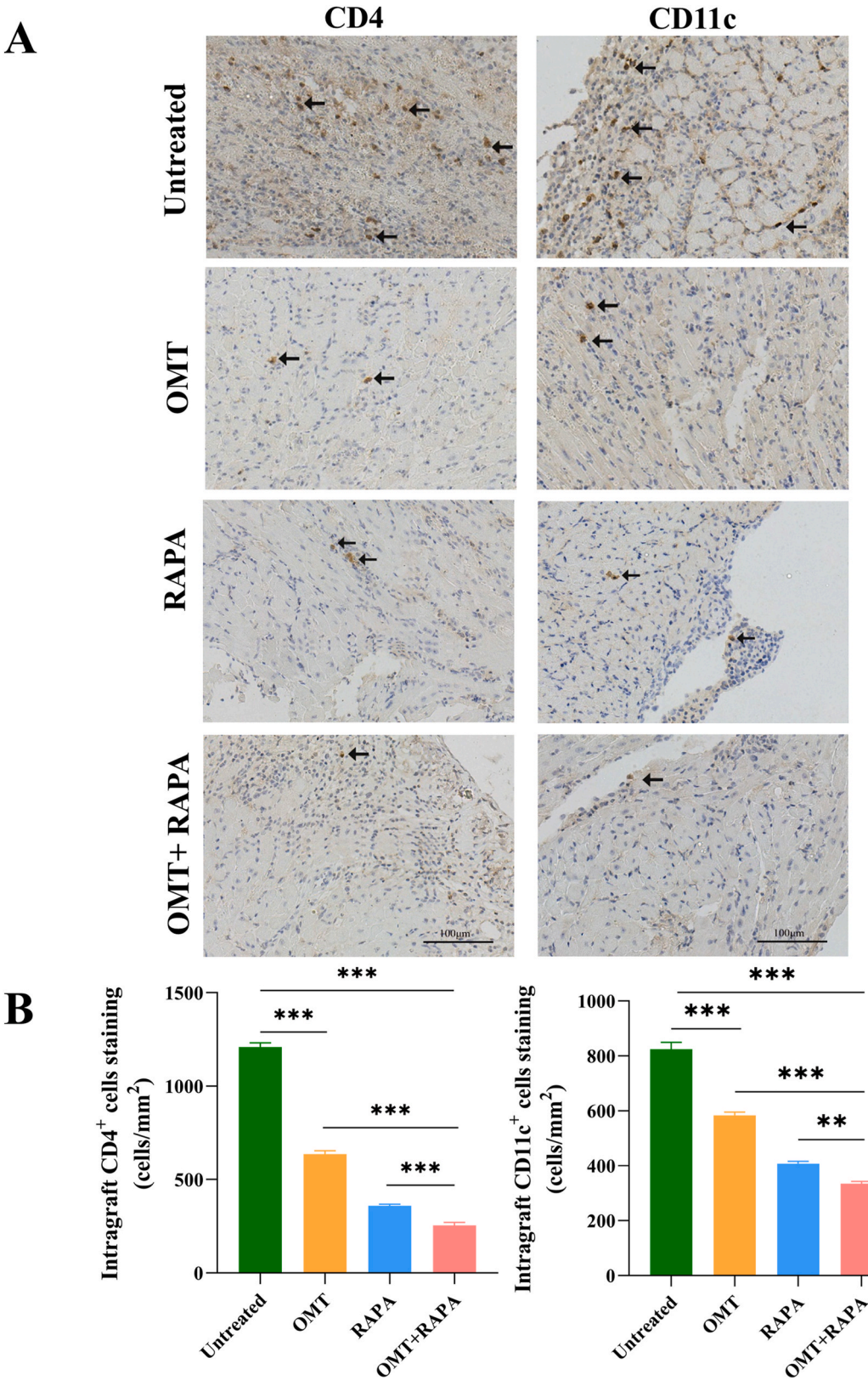

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Fig. 2. OMT decreased the infiltration of $CD4^+$ cells and $CD11c^+$ cells in cardiac allografts. (A) The cardiac allografts from each group were collected on postoperative day 8 and stained with either $CD4^+$ or $CD11c^+$ monoclonal antibodies (n = 6 per group). The arrows show positive staining. (B) The number of intragraft $CD4^+$ and $CD11c^+$ infiltrating cells in each group was presented by quantitating all the positive staining cells within a given section (cells/mm^2). Statistical analysis was done by one-way ANOVA followed by the LSD test (n = 6 per group), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.2. OMT in combination with RAPA decreased $CD4^+$ and $CD11c^+$ cell infiltration in cardiac allografts

Immunohistochemistry was performed to detect the infiltration of $CD4^+$ and $CD11c^+$ cells in cardiac allografts. As shown in Fig. 2A, a large number of $CD4^+$ and $CD11c^+$ cell infiltrates were seen in the untreated group. In the OMT-treated group, the numbers of

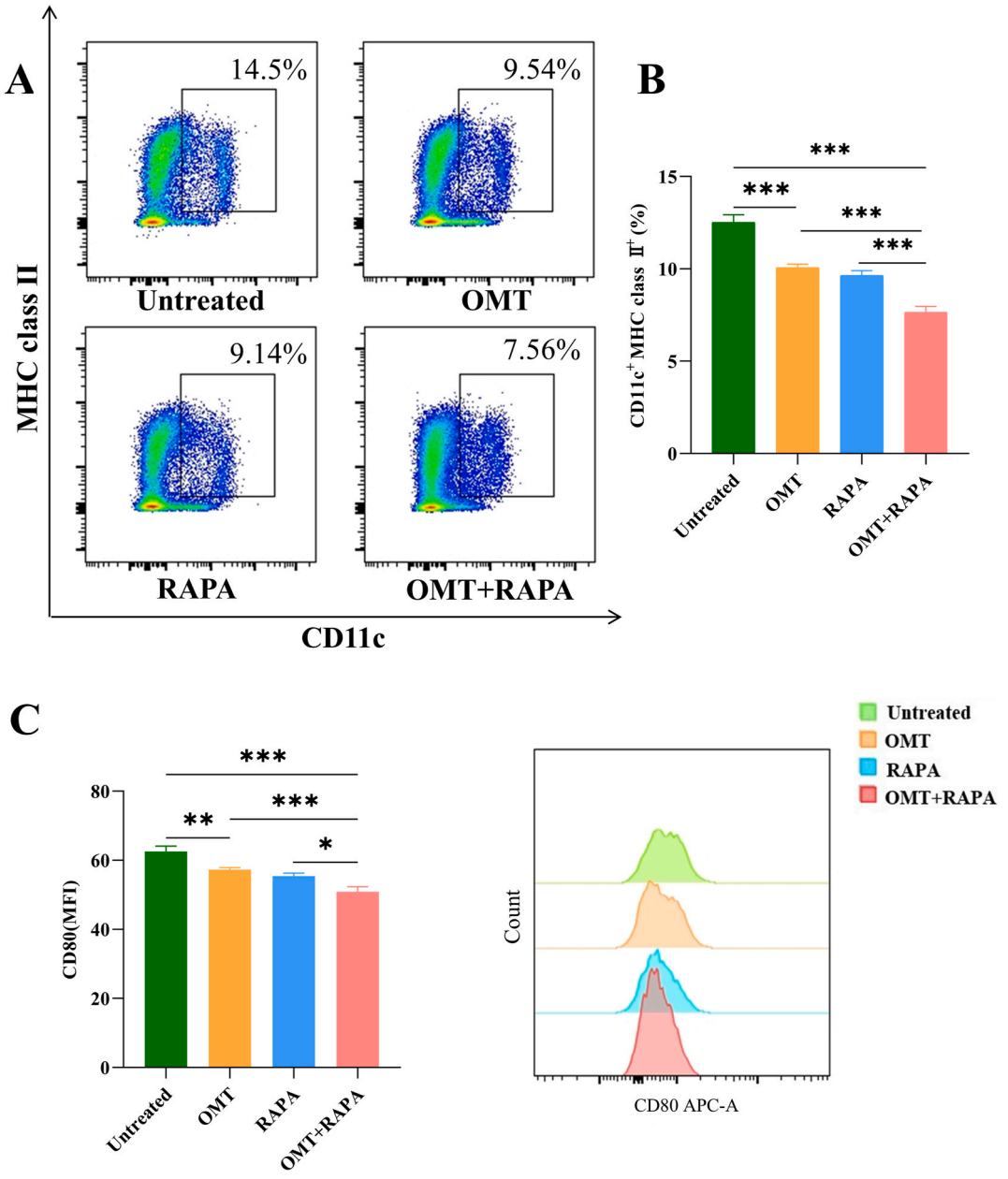

Fig. 3. OMT decreased the percentage of mature DCs in the spleen of transplant recipients. The splenocytes of C57BL/6 recipients were collected on postoperative day 8. The mature DCs were measured by anti-mouse CD11c, MHC class II, and CD80 antibodies. (A) Dot plots of $CD11c^+$ MHC class II^+. (B) Percentage of $CD11c^+$ MHC class II^+. (C) MFI and histogram graphs of $CD80^+$ which gating on $CD11c^+MHC\ II^+$ DC (n = 6). Statistical analysis was done by one-way ANOVA followed by the LSD test (n = 6 per group), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

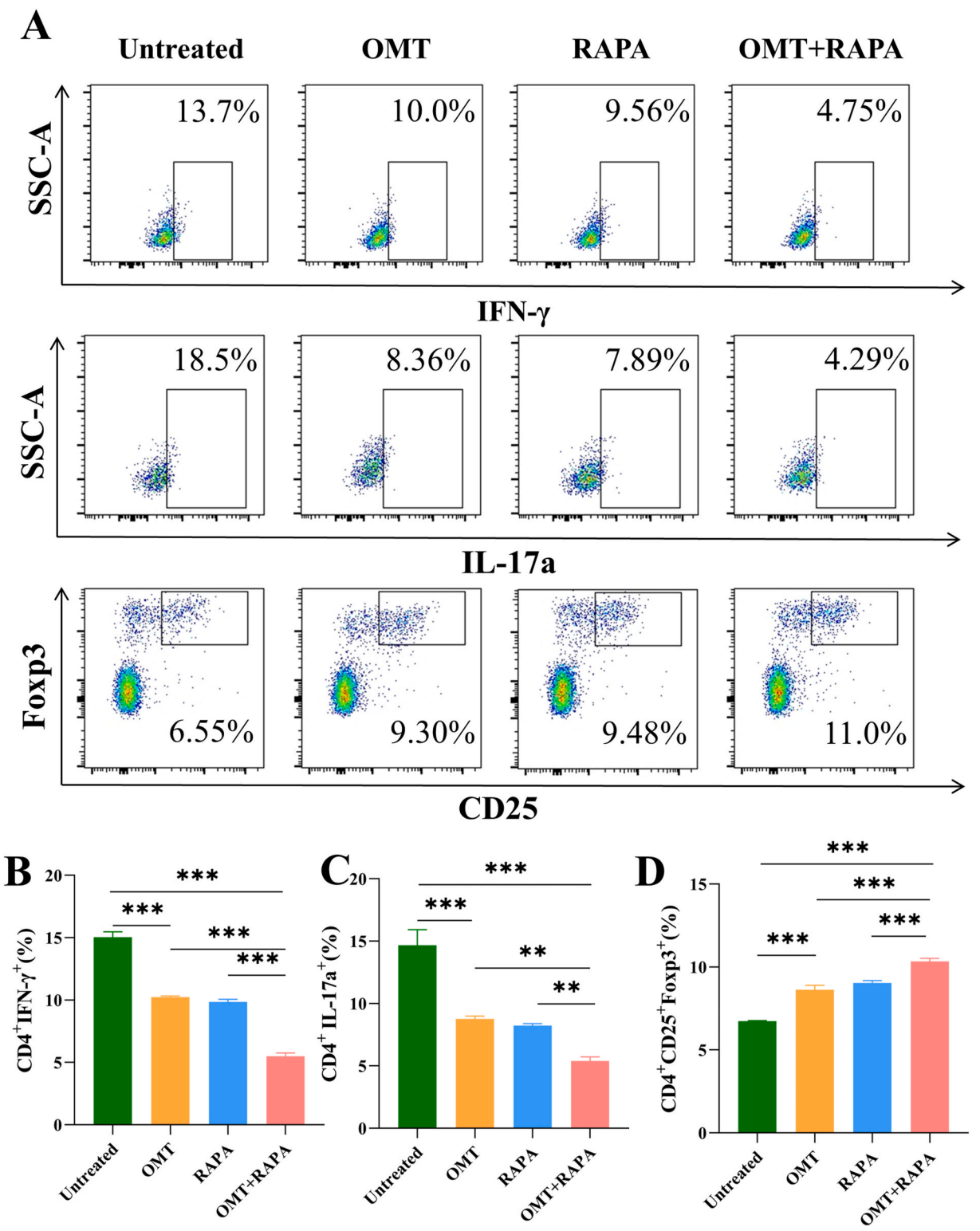

Fig. 4. OMT increased the percentage of Tregs and decreased the populations of Th1 and Th17 in transplant recipients. (A) The splenocytes of C57BL/6 recipients were collected at postoperative day 8, and the frequencies of Th1 (IFN-γ gated from $CD4^+$ T cells), Th17 (IL-$17a^+$ gated from $CD4^+$ T cells), and Treg ($CD25^+FoxP3^+$ gated from $CD4^+$ T cells) were analyzed by flow cytometry. The percentages of Th1($CD4^+IFN\text{-}\gamma^+$) (B), Th17 ($CD4^+IL\text{-}17a^+$) (C), and Treg ($CD25^+FoxP3^+$ gated from $CD4^+$ T cells) (D) in each group. Statistical analysis was done by one-way ANOVA followed by the LSD test (n = 6 per group), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

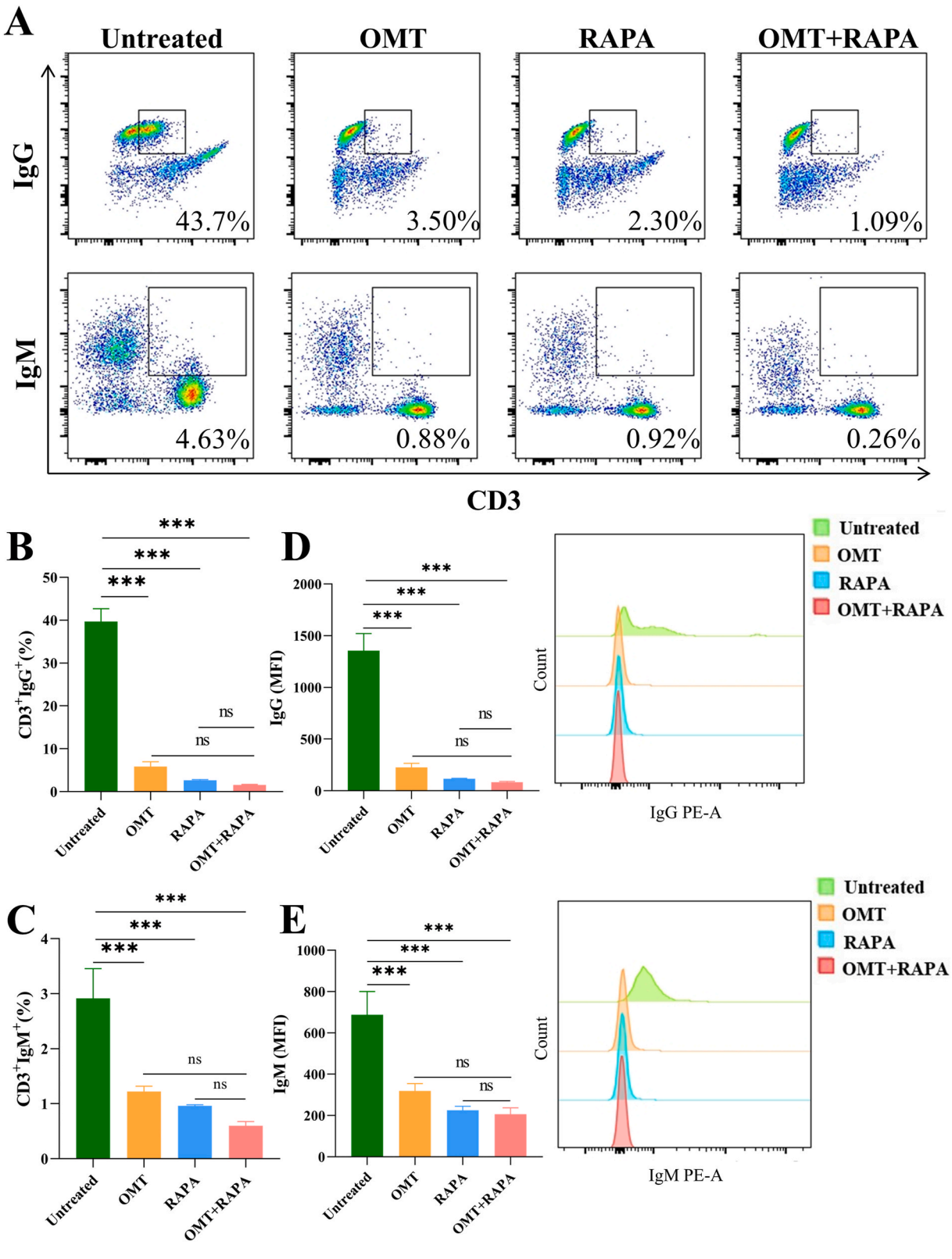

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Fig. 5. OMT therapy inhibited donor-specific antibody production. We measured donor-specific IgG and IgM by using the donor-derived splenocytes co-culturing with recipient serum (1:20 dilution) at 37 °C for 30 min. Then, donor $CD3^+$ T cell reactive IgG and IgM were measured by flow cytometry analysis respectively. (A) Dot plots of $CD3^+IgG^+$ and $CD3^+IgM^+$. (B) Percentage of $CD3^+IgG^+$ cells. (C) Percentage of $CD3^+IgM^+$ cells. (D) MFI, and histogram graphs of IgG in each group. (E) MFI, and histogram graphs of IgM in each group (n = 6). Differences among groups were assessed by using one-way ANOVA followed by the LSD test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

intragraft $CD4^+$ and $CD11c^+$ cells were significantly reduced compared with those of the untreated group (OMT-treated group *vs*. untreated group: $CD4^+$, $p < 0.001$; $CD11c^+$, $p < 0.001$; Fig. 2B). In addition, the inhibitory effect of combination therapy group was significantly improved compared with that of OMT or RAPA-treated group (OMT + RAPA treated group *vs*. OMT-treated group: $CD4^+$, $p < 0.001$; $CD11c^+$, $p < 0.001$, OMT + RAPA treated group *vs*. RAPA-treated group: $CD4^+$, $p < 0.001$; $CD11c^+$, $p < 0.01$; Fig. 2B). These results indicate that OMT alone effectively alleviates acute allograft rejection by reducing intragraft infiltration of $CD4^+$ and $CD11c^+$ cells, and in combination with RAPA displays a better therapeutic effect.

3.3. OMT in combination with RAPA reduced the population of mature DCs in the splenocytes of transplant recipients

As the high expression of MHC class II and CD80 on the surface of DCs presents the maturation of DCs and the efficient initiation of immune responses by DCs, we further investigated the effect of OMT either alone or in combination with RAPA on the expression of MHC class II and CD80 on DCs. The flow cytometry was used to detect the proportion of MHC class II+and $CD80^+$ DCs on POD8. As shown in Fig. 3A, OMT significantly reduced the proportion of both $CD11c^+$MHC class II^+DCs (OMT-treated group *vs*. untreated group: $p < 0.001$; Fig. 3B) and MFI of CD80 gating on $CD11c^+$MHC II^+ DCs (OMT-treated group *vs*. untreated group: $p < 0.01$; Fig. 3C). In addition, OMT in combination with RAPA further reduced the proportion of mature DCs in the splenocytes of transplant recipients (OMT + RAPA treated group *vs*. OMT-treated group: MHC class II, $p < 0.001$; CD80, $p < 0.001$; OMT + RAPA treated group *vs*. RAPA-treated group: MHC class II, $p < 0.001$; CD80, $p < 0.05$; Fig. 3B and C). These results show that OMT alone can effectively decrease the population of splenic mature DCs, and in combination with RAPA has a greater therapeutic effect in this process in transplant recipients.

3.4. OMT either alone or in combination with RAPA increased the proportion of tregs and decreased the populations of Th1 and Th17 cells in transplant recipients

Splenocytes of recipient mice were taken and analyzed by flow cytometry (Fig. 4A), and we found that the population of Th1 and Th17 cells were obviously decreased (OMT-treated group *vs*. untreated group: Th1 cells, $p < 0.001$; Th17 cells, $p < 0.001$; Fig. 4B and C), but Treg proportion was significantly increased in OMT-treated group (OMT-treated group *vs*. untreated group: $p < 0.001$; Fig. 4D). Furthermore, the combination therapy was superior to RAPA or OMT in inducing $CD4^+$ cell differentiation toward immunosuppression (OMT + RAPA treated group *vs*. OMT-treated group: Th1 cells, $p < 0.001$; Th17 cells, $p < 0.01$; Treg, $p < 0.001$; OMT + RAPA-treated group *vs*. RAPA-treated group: Th1 cells, $p < 0.001$; Th17 cells, $p < 0.01$; Treg, $p < 0.001$; Fig. 4B–D). These results suggest that OMT in combination with RAPA could further increase the percentages of Tregs and decrease the populations of Th17 and Th1 in transplant recipients to a certain extent.

3.5. OMT inhibited DSA production in transplant recipients

To determine the therapeutic effect of OMT either alone or in combination with RAPA, we measured serum levels of donor-specific IgG and IgM antibodies in transplant recipients. As shown in Fig. 5, the percentages of $CD3^+IgG^+$ and $CD3^+IgM^+$ cells (OMT-treated group *vs*. untreated group: IgG, $p < 0.001$; IgM, $p < 0.001$; Fig. 5A–C), as well as the level of IgG or IgM were significantly reduced in the OMT monotherapy group (OMT-treated group *vs*. untreated group: IgG, $p < 0.001$; IgM, $p < 0.001$; 5D and 5E), indicating that OMT monotherapy effectively downregulated DSA production in the recipients. Interestingly, The therapeutic effect of the combination treatment group on antibody levels was not statistically significant as compared with that of the RAPA or OMT monotherapy group (proportion of positive cells, OMT + RAPA-treated group *vs.* OMT-treated group: IgG, *ns*; IgM, *ns*; OMT + RAPA-treated group *vs.* RAPA-treated group: IgG, *ns*; IgM, *ns*; Fig. 5A–C)(MFI, OMT + RAPA-treated group *vs.* OMT-treated group: IgG, *ns*; IgM, *ns*; OMT + RAPA-treated group *vs.* RAPA-treated group: IgG, *ns*; IgM, *ns*; 5D and 5E). Given the above results, it suggests that OMT could effectively inhibit DSA production in transplant recipients.

3.6. OMT in combination with RAPA inhibited mTOR and HIF-1α expression in the allograft of transplant recipients

The expressions of p-mTOR and HIF-1α on $CD4^+$ cells and $CD11c^+$ cells in allografts were detected by flow cytometry. We extracted cells from cardiac allografts and detected the expression of p-mTOR and HIF-1α in both $CD4^+$ and $CD11c^+$ cells. The grafts were collected from three randomly selected recipient mice of each group. Due to the limited size of the grafts, mononuclear cells were extracted from a portion of each graft. We found that OMT significantly reduced the expressions of p-mTOR and HIF-1α in $CD4^+$ cells (OMT-treated group *vs*. untreated group: p-mTOR, $p < 0.01$; HIF-1α, $p < 0.001$; Fig. 6A), and in $CD11c^+$ cells (OMT-treated group *vs*. untreated group: p-mTOR, $p < 0.05$; HIF-1α, $p < 0.01$; Fig. 6B) in cardiac allografts compared with untreated group. While the combination therapy group further inhibited intragraft mTOR and HIF-1α expressions in $CD4^+$ or $CD11c^+$ cells as compared to RAPA-

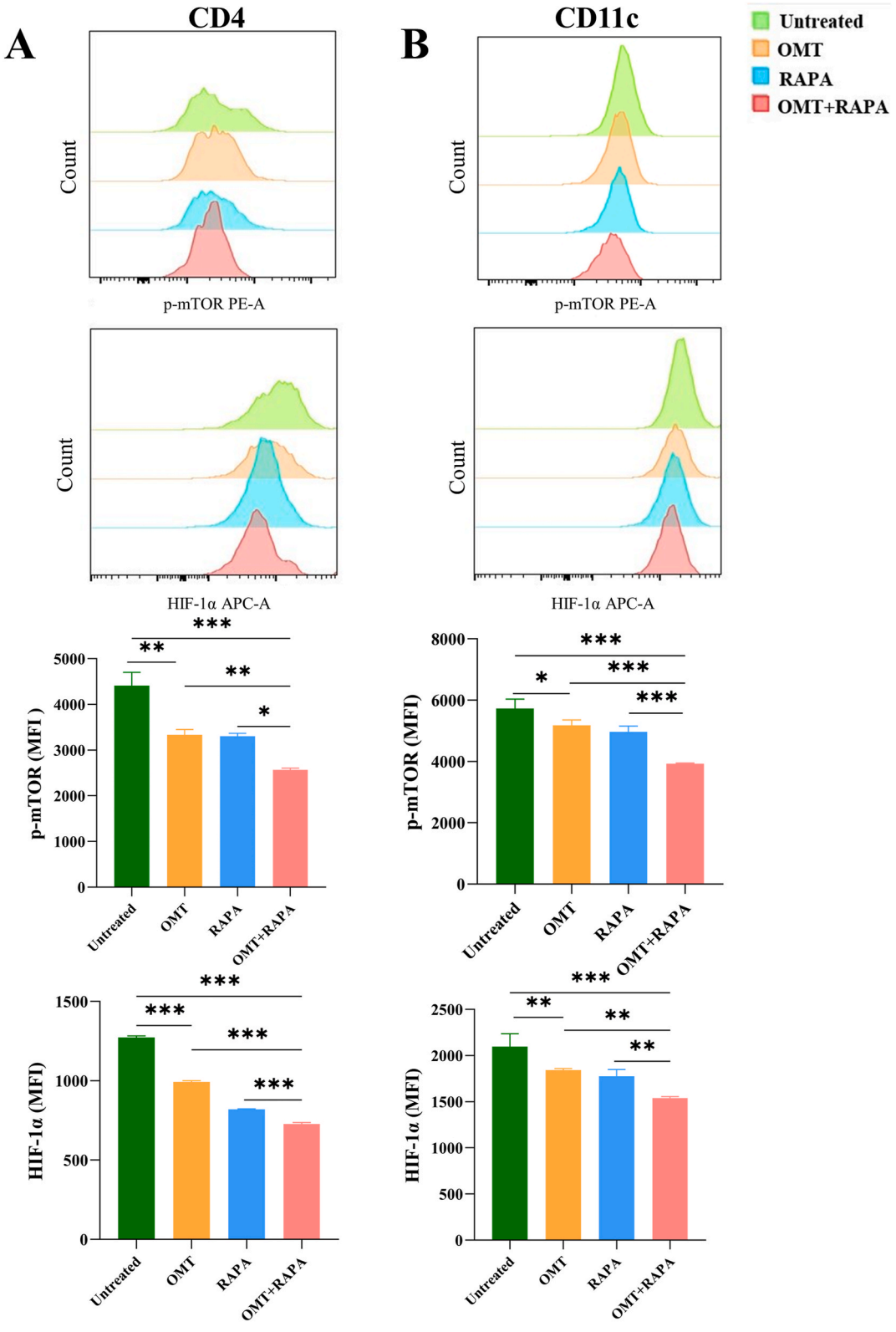

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Fig. 6. OMT inhibited mTOR and HIF-1α expression in the allografts of transplant recipients. The mononuclear cells were isolated from the grafts and analyzed by flow cytometry. The histogram graphs and MFI of intragraft $CD4^+$ cells ($CD4^+$p-$mTOR^+$ and $CD4^+HIF\text{-}1\alpha^+$) (A) and $CD11c^+$ cells ($CD11c^+$p-$mTOR^+$ and $CD11c^+HIF\text{-}1\alpha^+$) (B) were evaluated in cardiac allografts from different groups. Statistical analysis was done by one-way ANOVA followed by the LSD test (n = 3 per group), *p < 0.05, **p < 0.01, ***p < 0.001.

treated group or OMT-treated group ($CD4^+$ cells, OMT + RAPA-treated group *vs.* OMT-treated group: p-mTOR, $p < 0.01$; HIF-1α, $p < 0.001$; OMT + RAPA-treated group *vs.* RAPA -treated group: p-mTOR, $p < 0.05$; HIF-1α, $p < 0.001$; Fig. 6A) ($CD11c^+$ cells, OMT + RAPA-treated group *vs.* OMT-treated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.01$; OMT + RAPA-treated group *vs.* RAPA -treated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.01$; Fig. 6B). These data indicate that OMT effectively reduced p-mTOR and HIF-1α expression in $CD4^+$ cells and $CD11c^+$ cells, and this could be one of the mechanisms by which OMT exerts its graft-protective effect.

3.7. OMT inhibited mTOR and HIF-α expressions in $CD4^+$T cells in vitro

In this study, we performed *in vitro* experiments to detect the expression of p-mTOR and HIF-1α in $CD4^+$ T cells. The optimal therapeutic concentration of OMT was validated and selected at 0.5 mg/ml. The splenic $CD4^+$ T cells were isolated from C57BL/6 mice by magnetic bead sorting and divided into untreated, OMT-treated, OMT with the addition of MHY1485-treated groups and MHY1485 alone-treated group. Similar to the results of BMDCs, the expressions of p-mTOR and HIF-1α were markedly decreased in $CD4^+$ T cells treated with OMT compared to the untreated group (OMT-treated group *vs*. untreated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.001$; Fig. 7A), but MHY1485 inverted this situation (OMT-treated group *vs*. OMT + MHY1485 treated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.001$, OMT + MHY1485 treated group *vs*. MHY1485 alone-treated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.05$; Fig. 7A). The expressions of both p-mTOR and HIF-1α were increased under the influence of MHY1485. This suggests that OMT may achieve immunosuppression by affecting the differentiation of $CD4^+$ T cells, the process is associated with inhibiting the activation of mTOR and HIF-1α.

3.8. OMT inhibited mTOR and HIF-α expressions in BMDCs in vitro

We have previously demonstrated that OMT can inhibit the DC maturation of splenocytes *in vitro*. To further prove the *in vivo* finding on the mTOR–HIF–1α pathway and elucidate the molecular pathway targeted by OMT, we designed *in vitro* experiments in which the expression of p-mTOR and HIF-1α in BMDCs were stimulated by LPS under the influence of various conditions. In this study, we have validated the optimal concentration of OMT at 0. 5 mg/ml *in vitro*. We extracted BMDCs from C57BL/6 mice and divided them into untreated, OMT-treated, OMT with the addition of MHY1485 (mTOR activator)-treated and MHY1485 alone-treated group. Compared with the untreated group, p-mTOR and HIF-1α expression of BMDCs was significantly decreased in the OMT-treated group (p-mTOR, $p < 0.001$; HIF-1α, $p < 0.001$; Fig. 7B). However, MHY1485 blocked the function of OMT resulting in upregulations of p-mTOR and HIF-1α (OMT-treated group *vs*. OMT + MHY1485 treated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.01$; OMT + MHY1485 treated group *vs*. MHY1485 alone-treated group: p-mTOR, $p < 0.001$; HIF-1α, $p < 0.01$; Fig. 7B). The expressions of both p-mTOR and HIF-1α were significantly increased by activating the mTOR–HIF–1α pathway with MHY1485. This result suggests that OMT inhibits BMDC maturation by suppressing mTOR and HIF-1α activation.

4. Discussion

In our previous study, we reported for the first time that OMT can safeguard cardiac allografts and increase their survival period [34]. In the current study, we further demonstrated that OMT, either alone or in combination with RAPA, prolonged cardiac allograft survival, alleviated pathological damage, decreased the infiltration of $CD4^+$ and $CD11c^+$ cells in the grafts, and the percentages of mature DCs, Th1, and Th17 cells in splenocytes, but increased the percentage of Tregs. OMT-based therapy also decreased mTOR and HIF-1α in $CD4^+$ T or $CD11c^+$ cells in recipient spleens and cardiac allografts. Additionally, the *in vitro* study indicated that OMT suppressed the mTOR–HIF–1α pathway in BMDCs and $CD4^+$ T cells. Overall, our investigation suggested for the first time that OMT and RAPA have an additive effect on the attenuation of cardiac allograft rejection. OMT inhibited DSA production in transplant recipients. The mTOR–HIF–1α pathway plays an important role in OMT-based therapies.

RAPA is a clinical option for treating transplant rejection because it inhibits cell- and antibody-mediated rejection [33]. RAPA substantially reduced the maturation of DCs and modulated the differentiation of $CD4^+$ T cells toward immunosuppression. OMT and matrine (MT) are both extracted from ginseng and have very similar structures; however, OMT contains more than one oxygen atom at carbon C-1. Additionally, they have similar anti-inflammatory and antitumor effects [37,38]. Previous studies have demonstrated that MT and RAPA synergistically enhance apoptosis by inhibiting Akt/mTOR signaling, thereby reducing the burden of acute myeloid leukemia *in vivo* [39]. OMT protects cardiac grafts and substantially prolongs allograft survival. Therefore, we supplemented OMT-based therapy by combining it with RAPA, which also inhibits mTOR, to explore whether the combination of the two drugs could achieve immunosuppression. Furthermore, we suggest that OMT in combination with RAPA is, in a similar way, capable of prolonging the effects of RAPA by inhibiting the mTOR–HIF–1α signaling pathway. The present study demonstrated that the combination of OMT and RAPA substantially improved the survival time of allografts and was superior to monotherapy with OMT or RAPA alone.

For decades, it was believed that acute transplant rejection was mostly caused when recipient $CD4^+$ T cells recognized intact MHC class II alloantigens on the surface of donor APCs, specifically DCs [40]. *In vivo*, treatment with OMT and/or RAPA resulted in a

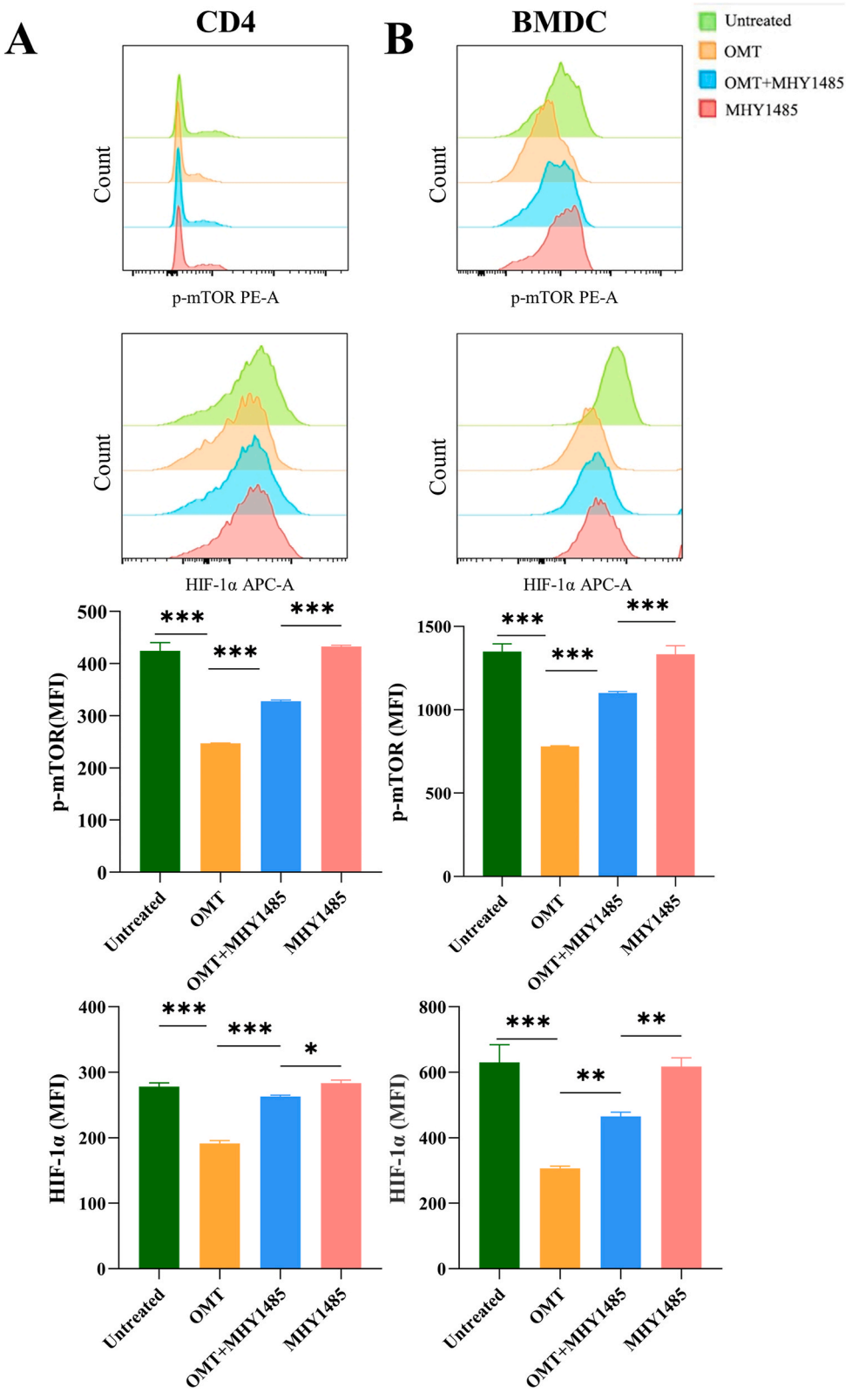

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Fig. 7. OMT inhibited the expression of mTOR–HIF–1α in $CD4^+$ T cells and BMDCs. BMDCs were induced from bone marrow cells of C57BL/6 mouse and naive $CD4^+$ cells were isolated from splenocytes of C57BL/6 mouse by CD4 microbeads kit *in vitro*. BMDCs and $CD4^+$ cells from different groups were treated with vehicle control, untreated group (add LPS or stimulator), OMT 0.5 mg/ml (add LPS or stimulator + OMT), OMT + MHY1485 (add LPS or stimulator + OMT + MHY1485) and MHY1485 (add MHY1485). The expression of mTOR–HIF–1α in $CD4^+$ cells or BMDCs was measured by flow cytometry. The histogram graphs and MFI of intragraft $CD4^+$ cells ($CD4^+$p-$mTOR^+$ and $CD4^+HIF\text{-}1\alpha^+$) (A) and $CD11c^+$ cells ($CD11c^+$p-$mTOR^+$ and $CD11c^+HIF\text{-}1\alpha^+$) were evaluated from different groups. Statistical analysis was done by one-way ANOVA followed by the LSD test (n = 3 per group), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

considerable reduction in both the local (in the cardiac allograft) and systemic (in the recipient spleen) levels of DCs and $CD4^+$ T cells. We also found that the maturation of DCs and differentiation of $CD4^+$T cells into Th1 or Th17 cells were substantially inhibited by OMT *in vitro*. Current research on the involvement of OMT in DC regulation is limited. OMT reduces acute intestinal inflammation by preventing the generation of pro-inflammatory cytokines, such as IL-1β induced by lipopolysaccharide (LPS) in BMDCs [41]. Naive $CD4^+$ T cells first develop into Th0 cells after antigen stimulation and subsequently transform into Th1 and Th17 cells [42]. Th1 and its hallmark secretory factor IFN-γ are critical in initiating an inflammatory response, which contributes to the development of acute transplant rejection [43]. IFN-$\gamma^{-/-}$ mice have substantially longer cardiac allograft survival than wild-type mice [44]. Th17 cells are closely associated with inflammation and promote inflammatory responses by secreting IL-17A [45,46]. $CD4^+$ T cell clones derived from renal allografts of patients with acute rejection produce high levels of Th1 cytokines (IFN-γ) after stimulation in clinical kidney transplantation, showing that alloreactive Th1 cells are implicated in the development of acute rejection [47]. Additionally, blocking IL-17A substantially lengthened the graft survival time in a rat cardiac transplantation model [48]. OMT downregulates Th1 and Th17 differentiation to alleviate ulcerative colitis and acute pancreatitis [17,18,49]. Strong evidence suggests that OMT is capable of inhibiting the production of T-bet and ROR-γt, a transcription factor unique to Th1 and Th17, and lowering levels of IFN-γ and IL-17A in L-arginine-induced acute pancreatitis model [49]. According to the results of our study, the inhibitory effects of OMT and/or RAPA on the maturation of DCs and the differentiation of $CD4^+$T cells into Th1 or Th17 cells are essential for the prevention of ACR.

Tregs are crucial for preserving immune balance and protecting allografts in transplant models [50]. DCs promote immunological tolerance in mice by generating antigen-specific Tregs [51]. OMT increased the percentage of Tregs and acted as an anti-inflammatory and anti-pruritic agent in a mouse model of allergic contact dermatitis [13]. In addition, OMT's anti-inflammatory effects on rheumatoid arthritis on rat RA were achieved by increasing the proportion of Tregs and decreasing the proportion of Th17 cells [52]. In this study, we analyzed the effects of combination therapy on Treg population regulation. Transplant recipients treated with OMT alone showed a substantial increase in the proportion of splenic Tregs, which was further enhanced by combined OMT and RAPA treatment. This suggests that OMT, in combination with RAPA, prevents the development of cardiac allograft rejection, and this process is associated with the enhancement of Tregs.

DCs present intact antigens that are recognized by B cells [53], directly activate B cells [54], and initiate antibody responses [55]. Upon co-culturing B cells with antigen-specific $CD4^+$ T cell lines, naïve B cells secreted both IgM and IgG after co-culturing with antigen-loaded DCs. However, only IgM secretion was observed when B cells were co-cultured with antigens in the absence of DCs. These studies indicated that DCs have a substantial role in the initiation and regulation of antibody synthesis [9]. IgG also increases proliferation and activation of antigen-specific $CD4^+$ T cells [56–58]. Few studies have reported the effects of OMT on B cells. Our previous experiments demonstrated that OMT increases the proportion of Bregs in splenocytes *in vitro* and in recipient mice *in vivo* [16]**.** The effect of OMT on DSAs has not been previously investigated. We observed that OMT reduced the proportion of mature DCs, suggesting that OMT also plays a role in reducing the production of DSA. In this study, we found for the first time that OMT substantially reduced DSA production *in vivo*, suggesting that OMT also has a therapeutic effect on antibody-mediated graft rejection, although the addition of RAPA did not achieve further effects on DSA inhibition in this model. Therefore, further studies are needed to determine whether long-term use of OMT in combination with RAPA can reduce the production of DSA.

mTOR plays a pivotal role in both innate and adaptive immune regulation, and its effects, as well as those of RAPA and its analogs as anti-rejection medications, have been extensively studied for the modulation of immune cell functions in allograft transplantation rejection [59,60]. Administration of RAPA *in vivo* hampers the upregulation of DC costimulatory molecules, production of pro-inflammatory cytokines, and the allostimulatory capacity of T cells [61]. mTOR is crucial for Th17 cell differentiation both *in vitro* and *in vivo*. Compared with wild-type T cells, mTOR-deficient T cells failed to differentiate into Th17 cells under Th17-skewing conditions *in vitro* [27]. According to Sauer, the mTOR signaling pathway regulates Treg cells and the expression of Foxp3. Inhibition of the PI3K/AKT/mTOR pathway promotes the expression of Foxp3 and Treg-like genes and microRNA expression in CD4 + T cells [62]. Recently, the role of mTOR in organ transplantation has received widespread attention. Avila et al. found that mTOR-I serves as an effective humoral immune suppressant, reducing alloantibody production in transplant recipients, directly inhibiting allo-IgG1 B-cell production of alloantibodies, and delaying graft rejection in both low and high alloantibody producers [63]. Thus, mTOR signaling plays a critical role in regulating transplantation rejection by modulating the maturation and differentiation of immune cells and the production of antibodies. mTOR is one of the upstream regulators of HIF-1α. Some reports suggested that mTORC1 is a positive regulator of HIF1α. mTOR has a direct effect on HIF-1α stability, and the activated state of mTOR increases HIF-1α expression even under hypoxic conditions. Furthermore, mTOR can directly enhance HIF-1α transcriptional activity, thereby activating HIF-1α [64,65]. HIF-1α plays a crucial role in immune cell metabolism and function. LPS stimulation elevates HIF-1α protein and mRNA levels in DCs, and knocking down HIF-1α in LPS-stimulated DCs reduces the expression of co-stimulatory molecules, CD80 and CD86, diminishing their capacity to stimulate allogeneic T cell proliferation [66]. HIF-1α is a key regulator of T cell differentiation and immune effector functions, directly involved in regulating the TH17/Treg balance [30]. Shi shows that mTOR signaling is vital for inducing HIF-1α expression in Th17 cells, indicating the HIF-1α-dependent glycolytic pathway as an essential component of

mTOR-mediated Th17 and Treg cell differentiation [67]. In summary, the mTOR–HIF–1α pathway can influence transplant rejection by regulating immune cell differentiation and antibody production that is associated with transplantation immunity. In many disease models, OMT operates by inhibiting the expression of mTOR and HIF-1α. OMT can suppress the PI3K/Akt/mTOR signaling pathway, which induces autophagy and is beneficial against synovial sarcoma [68]. In addition, OMT exhibits a synergistic effect by augmenting the antineoplastic properties of oxaliplatin in colon carcinoma via the PI3K/AKT/mTOR signaling pathway [69]. We suggested that OMT may exert a protective effect on cardiac allografts by inhibiting the mTOR–HIF–1α pathway, which was further verified in our experiments. Our research has shown that the mTOR–HIF–1α pathway is suppressed in $CD4^+$ or $CD11c^+$ cells of cardiac allografts *in vivo*. Our *in vitro* studies were performed on BMDCs and $CD4^+$ T cells to see if OMT therapy suppresses the mTOR–HIF–1α pathway in BMDCs and $CD4^+$ T cells. In our current study, OMT substantially reduced the mTOR–HIF–1α pathway expression in BMDCs and $CD4^+$ T cells *in vitro* and drove their differentiation toward immunosuppression, but adding MHY1485 reversed the therapeutic effect of OMT. This suggests that OMT targets the mTOR–HIF–1α pathway to attenuate cardiac allograft rejection.

In our study, no drug-induced deaths occurred in mice. The efficacy and safety of OMT treatment *in vivo* substantially vary depending on the route of administration. In mice, the median lethal dose (LD50) values were 347.44 mg/kg for males and 429.15 mg/kg for females via intraperitoneal injection; 256.74 ± 573.6 mg/kg via intramuscular injection; and 144.2 ± 22.8 mg/kg via intravascular injection [70,71]. Sprague-Dawley rats showed a much higher LD50 898.22 mg/kg via intraperitoneal injection [52]. In clinical studies, the main side effects included nausea, diarrhea, rash, fever, and frequent urination; however, no severe adverse effects were apparent [16]. These studies confirmed the safety of OMT with reasonable use.

Overall, this study has investigated the immunosuppressive efficacy of OMT in a transplant model and demonstrated that OMT in combination with RAPA in attenuation of acute allograft rejection. However, our study does have certain drawbacks. For example, besides the mTOR–HIF–1α pathway, OMT may have therapeutic benefits through other signaling pathways which needs further elucidation. Whether OMT inhibits mTOR and HIF expression separately or inhibits HIF-1α expression via mTOR suppression requires more advanced experiments in our next step study.

5. Conclusion

In conclusion, our investigation has demonstrated for the first time that OMT and RAPA have an additive effect to attenuate cardiac allograft rejection, OMT inhibits both ACR and DSA production in transplant recipients, and that the therapeutic effect of OMT is associated with mTOR–HIF–1α pathway. Furthermore, in-depth studies that may provide additional insights into the use of OMT as a therapeutic strategy are warranted.

Ethics statement

This study was reviewed and approved by the Animal Care and Use Committee of Tianjin Medical University, with the approval number: Ethic No. IRB2022-DW-23.

Data availability statement

The data associated with our study had not been deposited into a publicly available repository. The raw data required to reproduce these findings cannot be shared publicly at this time as the data also forms part of an ongoing study. Data will be made available on request. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Xu Lan: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jingyi Zhang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Shaohua Ren:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation. **Hongda Wang:** Writing – review & editing, Validation, Formal analysis, Data curation. **Bo Shao:** Writing – review & editing, Validation, Formal analysis, Data curation. **Yafei Qin:** Writing – review & editing, Validation, Formal analysis, Data curation. **Hong Qin:** Writing – review & editing, Validation, Formal analysis, Data curation. **Chenglu Sun:** Writing – review & editing, Validation, Formal analysis, Data curation. **Yanglin Zhu:** Writing – review & editing, Validation, Formal analysis, Data curation. **Guangming Li:** Writing – review & editing, Validation, Formal analysis, Data curation. **Hao Wang:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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