Targeting the class IA PI3K isoforms p110α/δ attenuates heart allograft rejection in mice by suppressing the CD4⁺ T lymphocyte response

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Abstract: Acute rejection is the most important factor causing allograft loss, which remains a challenge for patients undergoing organ transplantation. There is considerable evidence indicating that the activity of PI3K and its downstream positive and negative regulators plays a major role in regulating the activation of different subsets of effector CD4⁺ T cells. Thus, we investigated whether class A PI3Ks are involved in the development of acute allograft rejection, we found that p110α protein expression levels in the allograft group were significantly up-regulated on day 7 post-transplantation, while p110β and p110δ expression was significantly increased on days 5 and 7 post-transplantation. Treatment with PIK and IC but not TGX significantly prolonged allograft survival and altered pathological grades. The percentages of Th1 and Th2, Th17 and Tfh cells/monocytes in the spleens from the IC treatment group were all down-regulated. In contrast, the percentage of Treg cells in the spleens from IC treatment group was remarkably increased. IL-17A and IL-21 and IFN-γ expression levels were significantly decreased in the IC group. Moreover, IC significantly reduced P70 S6 Kinase β and 4E-BP1 protein expression. In conclusion, small-molecule inhibitors of p110δ and p110α suppress acute heart allograft rejection in mice. These inhibitors may play a role in anti-rejection by impacting the phosphorylation and expression of proteins in the AKT/mTOR pathway to modulate CD4⁺ T cell subsets levels in recipients, reduce proinflammatory factor expression and increase anti-inflammatory cytokine expression. These findings indicate that some small-molecule inhibitors of p110 can serve as novel targets in acute allograft rejection treatment.

Keywords: Heart transplantation, allograft rejection, class IA PI3K, CD4⁺ T cells, AKT/mTOR

Introduction

Acute rejection is the most important factor causing allograft loss, which remains a challenge for patients undergoing organ transplantation [1]. Several studies have demonstrated that the development of acute allograft rejection is associated with a poor prognosis [2]. The prevention and treatment of acute allograft rejection currently depends mainly on immunosuppressive agents, and the diagnosis of acute allograft rejection relies on pathological examinations of tissue allografts. Some immunosuppressants, such as the calcineurin inhibitors (CNIs), tacrolimus and cyclosporine A, have been shown to efficiently and potently inhibit acute rejection following transplantation; however, these treatments often fail to provide satisfactory results because they have numerous side effects, such as renal toxicity and neurotoxicity [3]. Thus, great effort has been made to identify novel and safe methods of preventing acute rejection.

Despite the performance of numerous medical and scientific studies in past decades, the mechanisms underlying allograft rejection remain unclear. T cells, especially CD4⁺ T cells, are key components of the adaptive immune system and play a central role in graft rejection following solid organ transplantation [4]. CD4⁺ T cell subsets, including the three major effector T helper (Th) cell lineages (Th1, Th2 and Th17), regulatory T cells (Tregs) and T follicular helper (Tfh) cells, are all involved in allograft rejection. Th1 responses initiate allograft rejection by promoting cytotoxic T cell activity and interferon-γ (IFN-γ)-mediated delayed-type
The expression of the class IA catalytic subunit p110 in cardiac grafts. Cardiac tissue samples were collected at the indicated time after transplantation. p110α, β and δ protein expression in the grafts was determined by western blotting on day 5 or 7 after transplantation. Each group (n = 3).

hypothesis that class A PI3Ks play an important role in the development of acute allograft rejection, which is likely mediated by CD4+ T cells. To validate this hypothesis, we established the acute rejection model of heterotopic cardiac transplantation in mice, according to a protocol described previously [24], and detected the expression of the class IA PI3K catalytic subunits in cardiac grafts. Furthermore, we investigated whether class IA PI3K isoform inhibitors can selectively lengthen allograft survival times and attenuate acute rejection by regulating different Th cell subsets.
and suppressing the CD4+ T lymphocyte response.

**Results**

The class IA catalytic subunit p110 protein was overexpressed in cardiac allografts

We successfully established the acute rejection model of heterotopic cardiac transplantation in mice. Histological analysis of cardiac allografts harvested on days 5 and 7 after allogeneic cardiac transplantation revealed that the grafts displayed several pathological features of acute rejection, findings consistent with those of a previous report [25]. The allografts displayed inflammatory cell infiltration and signs of destruction, whereas the isografts with which the allografts were compared displayed limited interstitial infiltration and tissue damage (not shown in the figure). p110α levels were...
To investigate the effect of class IA PI3K inhibition on acute rejection, we performed BALB/c-to-C57 mouse cardiac transplantation, after which we intraperitoneally injected the recipients with PIK-75 (PIK, 10 mg/kg/day), TGX-221 (TGX, 10 mg/kg/day), IC-87114 (IC, 15 mg/kg/day), or 10% dimethyl sulfoxide (DMSO, 0.5 ml/day/mouse; control) on the day of the operation and on days 2, 4 and 6 post-operation (Figure 2A). We found that treatment with PIK and IC significantly prolonged allograft survival in the corresponding treatment groups compared with the DMSO group (mean survival time [MST], 12.9±1.1, 14.8 versus 6.9 days; P<0.01; Figure 2B); however, treatment with TGX barely prolonged allograft survival in the TGX group compared with the DMSO group (MST, 7.7±1.7 versus 6.9 days; P>0.05; Figure 2B). To assess the effects of these inhibitors on the development of acute rejection-related pathology, we analyzed the graft tissue sections for signs of parenchymal rejection (PR) and graded PR severity in each group (Table 1). Hematoxylin-eosin staining revealed that the allografts from the PIK and IC groups displayed reduced inflammatory infiltration in the myocardium and less necrotic cardiomyocytes compared with the allografts from the DMSO and TGX groups (P<0.05; Figure 2C). TGX was unable to significantly alleviate acute rejection in the TGX group compared with the DMSO group (P<0.05; Figure 2C). Consistent with these findings, the PR scores of the allografts were significantly lower in mice treated with PIK and IC than in mice treated with DMSO or TGX at 7 days after transplantation (P<0.01; Figure 2D).

Class IA PI3K inhibitors modulated the percentages of splenic CD4+ T cell subsets in allograft recipients

The spleen, which is rich in lymphocytes, is involved in allograft rejection; thus, we assessed the impact of treatment with p110 inhibitors on the percentages of splenic CD4+ T cell subsets after cardiac transplantation. Mononuclear cells were isolated and then analyzed by flow cytometry, which showed that the percentages of Th1 (P<0.05; Figure 3A, 3D) and Tfh (P<0.05; Figure 4A, 4C) cells/monocytes in the spleens from the PIK group were lower than the percentages of Th1 and Tfh cells/monocytes in the spleens from the DMSO group (P<0.05). PIK treatment did not change the percentages of Th2 and Th17 cells/monocytes (Figure 3B, 3C, 3E, 3F) or the percentage of Tregs (Figure 4B, 4D) in the CD4+ T cell population of the PIK treatment group compared with those in the CD4+ T cell population of the control group. Similarly, the percentages of Th1 (P<0.01; Figure 3A, 3D), Th2 (P<0.05; Figure 3B, 3E), Th17 (P<0.05; Figure 3C, 3F) and Tfh (P<0.05; Figure 4A, 4C) cells/monocytes in the spleens from the IC treatment group were all lower than those in the spleens from the DMSO group; however, the percentage of Tregs (P<0.01; Figure 4B, 4D) was remarkably increased in the IC treatment group compared with control group. However, TGX did not change the percentages of Th1 (Figure 3A, 3D) and Th17 (Figure 3C, 3F) cells/monocytes or the percentage of Tregs (Figure 4B, 4D) in the CD4+ T cell population but reduced the percentages of Th2 (P<0.05; Figure 3B, 3E) and Tfh (P<0.05; Figure 4A, 4C) cells/monocytes in the spleens from the TGX group compared with those in the spleens from the control group after cardiac transplantation.

Class IA PI3K inhibition altered cytokine levels in the plasma of recipients

To determine whether these PI3K inhibitors suppressed the production of CD4+ T-related cytokines, we determined the concentrations of IL-17A, IFN-γ, IL-10, IL-21 and TGF-1 in the plasma of the recipients with ELISA. The data

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<th>Groups</th>
<th>Graft rejection scores</th>
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<tr>
<td>DMSO</td>
<td>3 4 2 3 3</td>
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<tr>
<td>PIK</td>
<td>2 2 2 1 1</td>
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Table 1. Graft rejection scores in the groups after 7 days

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were significantly lower in mice treated with PIK and IC than in mice treated with DMSO or TGX at 7 days after transplantation (P<0.01; Figure 2D).
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showed that IFN-γ levels were significantly lower in the allografts of mice treated with PIK (P<0.01), TGX (P<0.05) and IC (P<0.01) than in the allografts of mice treated with DMSO at 7 days after transplantation (Figure 5A). Similarly, the three inhibitors also reduced IL-17A levels in each treatment group compared with the control group (P<0.05, Figure 5C).

Figure 3. Class IA PI3K inhibitors reduced Th1, Th2 and Th17 cell/monocyte levels in the spleen. Splenocytes were obtained from allograft recipients in each group on day 7 after transplantation (A-C) The splenocytes were isolated and then stained with FITC-anti-CD4 before being activated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 mg/mL) in the presence of Brefeldin A (10 mg/mL) and Monensin (50 mg/mL). Finally, the splenocytes were intracellularly stained with PerCP Cyanine5.5-anti-IFN-γ, PE-anti-IL-4 and PE-anti-IL-17A before being analyzed by flow cytometry. The numbers indicate the percentages of Th1 (CD4+ IFN-γ+), Th2 (CD4+ IL-4+) and Th17 (CD4+ IL-17A+) cells in the monocyte population. (D-F) The percentages of Th1, Th2, and Th17 cells in the total monocyte population are shown as histograms. Data are shown as the mean ± SD for each group (n = 4). *P<0.05; **P<0.01.

DMSO, dimethyl sulfoxide; PIK, PIK-75; TGX, TGX-221; IC, IC-87114; IFN, interferon; IL, interleukin.
IL-10 levels in the TGX group compared with the control group (Figure 5B). Similar to IFN-γ and IL-17A levels, IL-21 levels were significantly decreased in the PIK (P<0.01), TGX (P<0.01) and IC (P<0.05) treatment groups compared with the DMSO group (Figure 5D). Interestingly, only the IC inhibitor increased the concentration of TGF-β, as there was no difference in TGF-β concentrations between the PIK and DMSO groups, nor was there a difference in TGF-β concentrations between the TGX and DMSO groups (Figure 5E).

Effect of class IA PI3K inhibitors on PI3K/AKT/mTOR pathway protein phosphorylation

The effects of PIK, TGX and IC on PI3K/AKT/mTOR pathway protein phosphorylation in car-

Figure 4. Class IA PI3K inhibitors altered Tfh and Treg cell levels in the spleen. Splenic T cells were obtained from allograft recipients in each group on day 7 after transplantation. A. The splenocytes were isolated and then stained with FITC-anti-CD4 and APC-anti-CXCR5, after which they were analyzed by flow cytometry. B. The splenocytes were isolated and then stained with FITC-anti-CD4 and PE-anti-CD25, after which they were intranuclearly stained with APC-anti-Foxp3 and analyzed by flow cytometry. The numbers indicate the percentages of Tfh (CD4+ CXCR5+) cells in the monocyte population and the percentages of Treg (CD4+ CD25+ Foxp3+) cells in the CD4+ T cell population. C, D. The percentage of Tfh cells in the monocyte population and the percentage of Treg cells in the CD4+ T cell population are shown as histograms. Data are shown as the mean ± SD for each group (n = 4). *P<0.05; **P<0.01. DMSO, dimethyl sulfoxide; PIK, PIK-75; TGX, TGX-221; IC, IC-87114; Tfh, follicular T-helper-cell; Treg, regulatory T cell.
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Figure 5. Class IA PI3K inhibitors altered cytokine levels in the plasma of the recipients. Blood was sampled from the eyes of allograft recipients in each group on day 7 after transplantation, after which it was centrifuged to obtain plasma for detection. IFN-γ (A), IL-10 (B), IL-17A (C), IL-21 (D) and TGF-β (E) concentrations in the sera of the recipients were assayed with ELISA (n = 6). *P<0.05, **P<0.01; DMSO, dimethyl sulfoxide; PIK, PIK-75; TGX, TGX-221; IC, IC-87114; IFN, interferon; IL, interleukin; TGF, transforming growth factor.

Figure 6. Effects of class IA PI3K inhibitors on PI3K/AKT/mTOR pathway protein phosphorylation in cardiac grafts. Cardiac allografts were harvested from each group on day 7 after transplantation. Analyses of the phosphorylation and expression of different proteins belonging to the PI3K/AKT/mTOR pathway were performed in (A and B). The western blotting results are representative of at least three independent experiments.
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Discussion

The results presented in this study demonstrate that class IA PI3K isoforms are involved in allograft rejection in donor hearts and that class IA PI3K inhibitors selectively prolong murine cardiac allograft survival. In this study, we investigated the possible mechanisms underlying the effects of class IA inhibitors on allograft rejection and survival. It is well known that acute allograft rejection is dependent on T cell subsets, including a variety of CD4+ and CD8+ T cell subsets [26, 27]. The contributions of alloreactive CD4+ T-cell subsets to acute allograft rejection were defined predominately by Bradley [28], who showed that adoptively transferred naive CD4+ T cells but not naive CD8+ T cells induced rapid rejection in a murine kidney transplant model at the time of transplantation. The PI3K signaling pathway, which has been studied mainly in tumor models, has recently emerged as a key molecular regulator of CD4+ T cell differentiation and function [18]. Superior PI3K protein expression and activity have been noted in most tumors, including glioblastoma and colorectal and lung cancer [29]. High p110 expression levels have been documented in some solid tumor cell lines; however, the functional role of p110 is unknown. p110 may be linked to tumor proliferation and growth [30]. We determined the expression of the class IA catalytic subunit p110α in cardiac grafts. The results showed that p110α was overexpressed in the rejection group compared with the syngeneic group on days 5 and 7 post-transplantation; however, p110α expression was increased in the rejection group compared with the syngeneic group only on day 5 after transplantation (Figure 1). The rejection allografts displayed more extensive CD4+ and CD8+ T cell infiltration than the isografts (not shown in Figure 1). These data are consistent with those from previous studies showing that p110β and p110α are ubiquitously expressed, whereas p110δ is highly enriched in leukocytes [17, 20, 29]. We speculated that p110 overexpression is caused by immunocyte infiltration and inflammatory reactions.

Many small-molecule inhibitors of class IA PI3Ks have been shown to have a negative effect on CD4+ T cell-related responses in vivo or in vitro [20, 23, 31]. Do some of these inhibitors suppress the rejection response in a similar way? To address this question, we attempted to investigate the therapeutic efficacy of the following inhibitors in allograft rejection: PIK-75 (selectively suppresses p110α), TGX-211 (selectively suppresses p110β) and IC-87114 (selectively suppresses p110δ). We found that PIK and IC significantly prolonged allograft survival in the corresponding treatment groups compared with the control group; however, TGX did not improve allograft survival in the TGX group compared with the control group. Hematoxylin & eosin staining showed that less inflammatory infiltrates and necrotic cardiomyocytes were present in the PIK and IC groups than in the control group; however, treatment with TGX did not affect inflammatory cell infiltration or cardiomyocyte necrosis. Similar results were noted with respect to the PR scores for the allografts on day 7 after transplantation (Figure 2). The low-rejection groups displayed limited CD4+ and CD8+ T cell infiltration compared with the control group (data not shown). Interestingly, Aragoneses-Fenoll [31] revealed that a p110α/δ inhibitor alleviated collagen-induced arthritis by modulating CD4+ T cell activation, and Acosta YY [22] reported that PIK-75, as a specific antagonist of p110p, ameliorated EAE by inhibiting CD4+ T lymphocyte activation. We suspected that the abovementioned mitigation of rejection may be related to the ability of PIK and IC to suppress T cell infiltration by modulating the expression and activity of CD4+ T cell subsets.

The spleen is an important immune organ in mice, and splenectomy can prolong cardiac allograft survival [32]. We examined the percentages of CD4+ T cell subsets in the spleen lymphocyte population at 7 days after transplantation. The flow cytometric data showed that the percentages of Th1 and Tfh cells in the spleens from the PIK group were lower than those in the spleens from the control group; however, PIK had a limited effect on the percentages of Th2, Th17 and Treg cells in the spleens from the PIK group. Unexpectedly, the percentages of Th1, Th2, Th17 and Tfh cells in the spleens from the IC treatment group were decreased compared with those in the spleens from the control group; however, the percentage of Treg cells in the spleens from the IC
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treatment group was remarkably increased compared with that in the spleens from the control group. TGX had little effect on the percentages of Th1, Th17 and Treg cells; however, TGX significantly decreased the percentages of Th2 and Tfh cells in the spleens from the TGX group compared with those in the spleens from the control group after cardiac transplantation. Previous studies showed that animal models of acute allograft rejection displayed increases in the expression of IFNγ mRNA, strongly suggesting that Th1 cells participate in allograft rejection. A growing body of literature suggests that Th17 cells play a role in the development of allograft rejection through an IL-17-mediated escape mechanism, particularly in cases in which Th1 responses are suppressed with CNIs [33], while Th2 responses have been suggested to delay acute allograft rejection, perhaps by downregulating Th1 cytokine production [24]. Tregs are fundamental for maintaining host immune tolerance after heterotopic transplantation [12, 26]. It was recently confirmed that effective humoral immunity depends on the support of B-cell responses induced by Tfh cells, which display C-X-C chemokine receptor type 5 expression and produce IL-21, as well as many other molecules (including programmed death-1, inducible costimulators and IL-4) [34-36]. Carla C. Baan [37] determined that IL-21 plays a key role in the production of Tfh cells in antibody-mediated rejection after organ transplantation. Based on these findings and our results, we surmised that IC alleviates acute allograft rejection by modulating the levels of CD4+ T cell subsets. Specifically, we hypothesized that IC reduced the percentages of potentially hazardous alloreactive T cells (Th1, Th2, Th17 and Tfh cells) but increased the percentages of immunosuppressive T cells (Tregs). We also surmised that PIK probably plays a role in suppressing rejection by decreasing the percentages of Th1 and Tfh cells. TGX has negligible effect on acute allograft rejection, namely, antibody-mediated rejection, by modulating the percentage of Tfh cells.

To investigate the effects of these inhibitors on CD4+ T-related cytokines further, we measured IL-17A, IFN-γ, IL-10, IL-21 and TGF-β concentrations in plasma. We found that all three p110 inhibitors, to varying degrees, suppressed IFN-γ, IL-17A and IL-21 expression in the plasma of recipients. Consistent with these findings, Acosta, Y. Y [22] found that PIK-75 (less than 10 nM) inhibited IFN-γ, IL-17A, and IL-21 secretion in activated CD4+ T blasts costimulated by ICOS, and Aragoneses-Fenoll, L [31] demonstrated that the in vitro activation of naive CD4+ T lymphocytes by anti-CD3 and anti-CD28 was inhibited more effectively by a p110δ inhibitor than by a p110α inhibitor, a finding supported by data pertaining to the secretion of cytokines (IL-2, IL-10, and IFN-γ), and that IC87114 inhibited Akt and Erk activation and IL-4, IL-17A, and IFN-γ secretion more effectively than A66 (a p110α inhibitor) in activated CD4+ T cells re-stimulated by CD3 and ICOS. Blanco [38] showed that all three inhibitors (PIK, TGX and IC) induced similar decreases in cytokine (IL-2, INF-γ, and IL-4) levels in culture supernatants. As previously reported, IFN-γ (secreted mainly by Th1 cells), IL-17A (secreted mainly by Th17 cells) and IL-21 (secreted mainly by Tfh and Th17 cells) play an important role in aggravating allograft rejection [4, 6, 26, 27, 32, 39]. Furthermore, PIK and IC but not TGX inordinately enhance IL-10 levels in recipients. Interestingly, only the IC group displayed a higher TGF-β level than the DMSO group. Based on our data (Figure 4B, 4D), we speculated that the changes in IL-10 and TGF-β levels were caused by increases in the percentage of Tregs, which secrete mainly anti-inflammatory cytokines [26, 27, 40]. Exogenous overexpression of immunomodulatory cytokines, such as IL-4, IL-10 and TGF-β, as well as the effects of inhibitors of pro-inflammatory cytokines, also delayed graft rejection [41]. Thus, we surmised that class IA PI3K inhibitors suppress the CD4+ T lymphocyte response by altering CD4+ T-related cytokine levels in the plasma of recipients by reducing pro-inflammatory factor levels or increasing anti-inflammatory cytokine levels.

Finally, to determine whether pharmacological inhibition of PI3K p110α/β/δ successfully attenuates basal AKT/mTOR signaling pathway activation, we determined the effects of PIK, TGX and IC on AKT/mTOR pathway protein phosphorylation in the corresponding allograft groups. The results showed that all three inhibitors reduced AKT phosphorylation but did not change total AKT expression (Figure 5). AKT, a key activator of mTOR, plays a role in cell cycle-related protein synthesis and activity. AKT activation promoted Th effector differentiation into Th1, Th2, and Th17 cells [18, 42];
however, studies have obtained contradicting results regarding the role of AKT in the peripheral differentiation of induced Tregs. For example, Haxhinasto. [43] reported that constitutive AKT activation impairs FOXP3 induction during in vitro TGF-β-driven Treg differentiation, suggesting that reduced AKT activity is required for peripheral Treg differentiation, as it is for natural Treg development. However, another study by Pierau. [44] found that in the absence of CD28 co-stimulation, AKT transgenic CD4+ T cells have an enhanced capacity to differentiate into Tregs. Perhaps a certain level of PI3K activity is necessary for maintaining Treg development and function, while excessive PI3K activity is detrimental with respect to Treg development and function. Diminished signaling was reflected not only by reduced AKT phosphorylation but also by changes in the levels or phosphorylation of downstream effectors, including the phosphorylation of p70 S6K and 4E-BP1. IC significantly reduced p70 S6K and 4E-BP1 protein expression, which probably resulted in diminished signaling. PIK but not TGX also reduced the expression of p70 S6K, and both agents had had limited effects on the expression of 4E-BP1. In summary, these three p110 protein inhibitors decrease the expression of non-identical components of AKT/mTOR signaling, such as AKT, p70 S6K and 4E-BP1. Importantly, activated mTOR phosphorylates and inhibits the eukaryotic initiation factor 4E-BP (4E-BP1, 2, 3) and activates the p70 S6 kinases (S6K1, 2), resulting in increased protein translation and glycolysis upregulation, promoting cell growth and division [42, 45]. Delgoffe. found that mTOR-deficient T cells, which displayed normal activation, failed to differentiate into Th1, Th2, or Th17 effector cells but differentiated into Foxp3(+) regulatory T cells under normal activating conditions [46]. Therefore, we speculated that IC and PIK suppress the AKT/mTOR signaling pathway to a greater extent than TGX by promoting the differentiation of CD4+ T cells into a state that benefits allografts in vivo.

Materials and methods

Animals

BALB/c (H-2d) and C57BL/6 (B6, H-2b) mice were used as donors and recipients, respectively (both strains of mice were aged 6–10 weeks and weighed 18-25 g at the start of the experiment; Beijing HFK Bioscience Co. Ltd, Beijing, China). All animals were maintained under controlled conditions (specific pathogen-free conditions, 22°C, 55% humidity and a 12-h day/night cycle) at the Animal Facility of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Heterotopic heart transplantation and post-transplantation treatments

Heterotopic cardiac transplantation was performed as described previously [24]. Cardiac graft pulsation was monitored daily by direct abdominal palpation, which was performed in a double-blinded manner, to determine whether the cardiac graft had survived or been rejected. The endpoint was complete cessation of cardiac contractility. PIK-75 (10 mg/kg/day), TGX-221 (10 mg/kg/day) or IC-87114 (15 mg/kg/day) was intraperitoneally injected into the recipients on the day of the operation and on days 2, 4 and 6 post-operation. All the class IA PI3K inhibitors used herein were purchased from Selleck, Houston, Texas, United States of America. The doses at which each inhibitor was administered were similar to those used in previous studies [22, 47, 48]. Recipients receiving 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA) served as controls. Allograft survival was reported as the MST ± SD.

Histologic analyses

The cardiac grafts, which were harvested from the recipients at 5 and 7 days after transplantation, were fixed in formalin and embedded in paraffin. The paraffin-embedded heart sections (4 µM) were processed for hematoxylin-eosin staining as described previously, and the degree of PR was graded as described previously [49] using the following scale: 0R = no rejection, 1R = focal mononuclear cell infiltrates without necrosis, 2R = focal mononuclear cell infiltrates with necrosis, 3R = multifocal infiltrates with necrosis and 4R = widespread infiltrates with hemorrhage and vasculitis.

Western blotting

p110 subunit and PI3K/AKT/mTOR pathway protein expression levels in the grafts were determined by western blotting. The cardiac
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Allografts were dissected, homogenized with lysis buffer (Cell Signaling, Beverly, MA, USA) on ice and then centrifuged at 16,000×g for 20 min at 4°C. The supernatants were collected and assayed for the total protein concentration. Protein concentration-normalized samples were subsequently electrophoresed and transferred onto PVDF membranes (Millipore, Bedford, MA, USA), which were blocked with 5% nonfat milk in Tris-buffered saline for 3 h and then incubated with the following primary antibodies overnight at 4°C: anti-PI3 Kinase p110β antibodies, anti-PI3 Kinase p110δ antibodies, anti-p-AKT1 (phospho S473) antibodies, anti-AKT1/2/3 antibodies, anti-P70 S6 Kinase β (phosphor S371) antibodies (all from Abcam Ltd, Cambridge, UK), anti-PI3 Kinase p110α antibodies, anti-P70 S6 Kinase β antibodies, anti-phospho-4E-BP1 (Thr37/46) antibodies, and anti-4E-BP1 (Thr46) antibodies (all from Cell Signaling Technology®, Leiden, the Netherlands). The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000) for 3 h. β-Actin or GAPDH was used as a loading control for comparisons between samples. The target protein bands were photographed with an ECL detection system (GE Health, Little Chalfont, UK).

Isolation of lymphocytes from spleens and flow cytometry

On day 7 post-operation, the recipients were sacrificed, and the spleens were harvested. Mononuclear cells were isolated from the spleens as described previously [24]. The proportions and numbers of specific types of lymphocytes in the spleens from the recipients were determined by flow cytometry, as described previously [4]. The cells were stained with FITC-anti-CD4, APC-anti-CXCR5 and PE-anti-CD25, after which they were stained with PerCP-Cy5.5-anti-IFN-γ, PE-anti-IL-4, PE-anti-IL-17A and APC-anti-FoxP3 (FITC-anti-CD4 was from BioLegend, San Diego, CA, USA; the remainder were from eBioscience, San Diego, CA, USA), according to the manufacturer’s protocols. Marker expression was assessed by flow cytometry using an FACS Calibur Flow Cytometer (BD Biosciences, San Diego, CA, USA), and the data were analyzed using FlowJo V10 software (Tree Star, Ashland, OR, USA).

ELISA

Serum IFN-γ, IL-10, IL-17A, IL-21 and TGF-β levels were measured by standard sandwich cytokine ELISA using cytokine ELISA kits, according to the manufacturer’s instructions (all kits were from MultiScience, Hangzhou, China).

Statistical analysis

Kaplan-Meier graphs were constructed to assess graft survival, and log-rank comparisons of the groups were performed to calculate P values. Data are presented as the mean ± SD, and comparisons of the values were performed using two-tailed Student’s t tests. All data were analyzed using Prism 5.0 (GraphPad Software, La Jolla, CA, USA). We considered P<0.05 statistically significant.

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Disclosure of conflict of interest

None.

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