Original Article
ITK inhibition promotes long-term survival of cardiac allografts by regulating T cell PLCγ phosphorylation

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Abstract: Background: T cells express interleukin-2 inducible T-cell kinase (ITK), which is an essential modulator of T-cell signaling and function. However, the role of ITK in solid organ transplantation has not been investigated to date. Here, we studied the function of ITK in a murine cardiac transplantation model. Method: Murine heart transplantation was performed using BALB/C mice as donors and C57BL/6 mice as recipients. Subsequent intraperitoneal injections of an ITK-specific inhibitor (BMS-509744) were performed to assess the effects of the kinase following cardiac transplantation. Additionally, naive T cells were isolated to investigate the inhibitor's potential effects in the alloimmune responses. Results: ITK inhibition was found to promote long-term cardiac allograft survival compared with the control group of 36.0 ± 3.8 days vs. 7.0 ± 0.7 days, respectively ($P < 0.01$). While the Th1/Th17 percentages showed a decrease in prevalence ($P < 0.001$), the CD4$^+$CD25$^+$Foxp3$^+$ percentages were not markedly affected. In vitro treatment of CD4$^+$ T cells with the ITK inhibitor downregulated the proliferation, possibly by regulating the phosphorylation of PLCγ. Conclusion: ITK inhibition resulted in lower Th1/Th17 responses after cardiac transplantation and markedly prolonged the mean survival time of the cardiac allografts. Thus, ITK inhibition might be a promising therapeutic target to alleviate alloimmune responses in the cardiac transplantation.

Keywords: Cardiac transplantation, interleukin-2 inducible T-cell kinase, transplant rejection

Introduction

The Tec family of tyrosine kinases plays a crucial role in lymphocyte development, activation, and differentiation [1]. The five main members of the Tec family are interleukin-2-inducible T-cell kinase (ITK), BMX non-receptor tyrosine kinase (BMX), Bruton's agammaglobulinemia tyrosine kinase (BTK), TXK tyrosine kinase (TXK, also RLK), and Tec protein tyrosine kinase (TEC) [1]. In T cells, the predominant Tec kinase is ITK, which acts downstream of the T-cell receptor (TCR) to regulate phospholipase C-γ (PLCγ) [2]. ITK signaling has been reported to regulate effector T cell differentiation and cytokine gene expression [3]. Thus, ITK exhibits an important role in the modulation of T cell signaling and function.

Early studies using primary murine ITK$^{-/-}$ T cells showed that ITK is required for robust T cell activation in response to TCR with co-stimulatory receptor signaling [3, 4]. ITK$^{-/-}$ mice showed greatly reduced CD4$^+$ T cells as well as affected Th1, Th2, and Th17 differentiation and cytokine production [4]. More specifically, ITK deficiency has been shown to skew the TCR response to Th1 differentiation [5], while ITK$^{-/-}$ mice revealed a lower activity in developing functional Th2 cells and showed resistance to allergen challenge [6]. Furthermore, studies indicated an important role of ITK in Th17 differentiation, and particularly ITK$^{-/-}$ T cells exhibited reduced production of IL-17A [4, 7, 8]. In addition, ITK$^{-/-}$ CD4$^+$ T cells developed higher percentages of functional Foxp3$^+$ cells and higher Foxp3 expression following in vitro polarization [7]. In

human studies, genetic deficiency and biochemical inhibition of ITK were found to affect Th17, Treg, and innate lymphoid cells [8].

The complex phenotype of ITK$^{-/-}$ mice, including defects in T cell development, activation, differentiation, and effector function, have made it difficult to precisely assess the function of ITK at different stages in each lineage of T cells of an immune response. Therefore, the use of ITK inhibitors (ITKi) such as BMS-509744 provides an alternative, practical strategy for the study of ITK both *in vivo* and *in vitro*. In addition to its highly selective inhibitory effects, BMS-509744 is not cytotoxic to human and mouse lymphocytes [9]. To date, no study has examined the relation between ITK inhibition and cardiac transplantation. This work aims to study the possibility of using ITKi as potential immunosuppressant to alleviate graft rejection following cardiac transplantation. Furthermore, we investigated and discussed the effects of ITKi in CD4$^+$ T cell-mediated alloimmune responses.

Materials and methods

Animals

All animals were kept under the guidance of the Care and Use of Laboratory Animals of the Laboratory Animal Ethical Commission of Tongji Medical College, Huazhong University of Science and Technology. Mice were aged 6-8 weeks, weighed 20-25 g, and were purchased from the Animal Center of Tongji Medical College. Male BALB/c mice were used as donors, and male C57BL/6 mice were used as recipients. The murine heterotopic heart transplantation model was conducted as previously described [10]. For the isograft group, male C57BL/6 mice were used both as donors and recipients. The recipients were divided into three groups (n = 5 for each group): (1) experiment group, treated after the operation with the ITK-specific inhibitor BMS-509744 (purchased from MCE, USA) 20 mg/kg injected intraperitoneally daily; (2) control and isograft group, administered with DMSO (Sigma-Aldrich, St. Louis, MO, USA) daily after transplantation. At the end of the experiment, animals were sacrificed, and spleens were collected for downstream analysis.

Cell preparation

The recipients were under Isoflurane anesthesia and surgical remove the spleen at each time point. Cells were isolated from the spleens as described previously [11]. Following spleen collection, 10 mL of cold RPMI medium (GE Healthcare) supplemented with 1% FBS was added, and red blood cells were lysed using the RBC lysis buffer (eBioscience). The resulting cell suspension was filtered with 40-µm meshes. Naive T cells from C57BL/6 mice were isolated using CD4$^+$ or CD8$^+$ microbeads (Miltenyi Biotec). Isolated cells were plated in 12- or 24-well plates and activated by plate-bound anti-CD3 (1.0 mg/mL) and anti-CD28 (2.0 mg/mL) antibodies (BD Biosciences).

Flow cytometry analysis

Lymphocyte suspension was obtained from the spleens of the recipients, as described above. The following monoclonal antibodies were used (all antibodies were purchased from BD Biosciences, San Diego, USA): FITC-anti-CD4, PE-CY7-anti-CD8, APC-anti-T-bet, PE-anti-Rorγt, PE-CY7-anti-Foxp3, FITC-anti-annexin V, PI, APC-anti-IFNγ, PE-anti-IL-17α, PE-CY7-anti-IL4, and PE-anti-CD45. For intracellular cytokine detection, cells were stimulated with a cell activation cocktail (2 µg/mL Brefeldin A) for 6 h prior to collection and staining. Marker expression was assessed by flow cytometry using a FACS Calibur Flow Cytometer II (BD Biosciences, San Diego, USA). Multi-color flow cytometric data analysis was performed using the Flowjo V10 software (Tree Star, Ashland, USA).

Real-time PCR

Allografts and spleens were processed for RNA extraction using the Trizol reagent (TaKaRa, Shiga, Japan), and cDNA was prepared by reverse transcription with the PrimeScript RT Master Mix (TaKaRa, Shiga, Japan). Real-time PCR was conducted on the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). Primer sequences are as follows. The mRNA expression levels of were measured by real-time qPCR. IFN-γ forward-GCCACGGCACAGTCATTGA, reverse-TGCTGATGGCCTGATTGTCTT. IL-2 forward-CCTGAGCAGGATGGAGAATTACA, reverse-TCCAGAACATGCCGCAGAG, IL-17A

forward-CTCCAGAAGGCCCTCAGACTAC, reverse-AGCTTTCCCTCCGCATTGACACAG, GM-CSF forward-GGCCTTGGAAGCATGTAGAGG, reverse-GGAGAACTCGTTAGAGACGACTT. T-bet forward-CAACAACCCCTTTGCGAAAG, reverse-TCCCCCA-AGCAGTTGACAGT. GATA-3 forward-AGCCACATC-TCTCCCTTCAG, reverse-AGGGCTCTGCCTCTCTA-ACC. RORγt forward-TGCAAGACTCATCGACAA-GG, reverse-AGGGGATTCAACATCAGTGC. Foxp3 forward-ACTGGGGTCTTCTCCCTCAA, reverse-G-TGGGAAGGTGCAGAGTAG.

Western blot

Cultured cells were directly lysed in sodium dodecyl sulfate (SDS) buffer and boiled for 10 min at 100°C for denaturation. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane for immunoblotting. Proteins were visualized using HRP-conjugated anti-mouse or anti-rabbit IgG and the ECL system (Amersham Biosciences). Total cell extracts were prepared in PBS and protease inhibitors. Protein concentrations were determined using the protein assay kit (eBioscience).

Statistical analysis

Data are shown as the mean ± SD. Graft survival was assessed using the Kaplan-Meier survival test. Statistical comparisons were performed using two-tailed Student's t tests. All data were analyzed using Prism 7 (GraphPad Software, La Jolla, USA). Values of $P < 0.05$ were considered statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Results

Inhibition of ITK prolonged the survival of cardiac allografts and impaired Th1/TH17 differentiation in vivo

To investigate the role of ITKi in alloimmune responses and establish a model of acute cardiac rejection, C57BL/6 mice were used as recipients for BALB/C donor hearts. Acute rejection was determined by histological analysis of the dense lymphocytic cellular infiltrate as well as vasculitis of organ donor vessels. Inhibition of ITK alleviated acute rejection (**Figure 1A**). The mean survival time of the allograft in the presence of ITKi was 36.0 ± 3.8 days, while it was around five times less in the control group (7.0 ± 0.7 days; $P < 0.01$; **Figure 1B**). Thus, compared with the DMSO group, the survival of the transplanted heart was significantly prolonged by ITKi (**Figure 1B**). The PR score of the allograft was significantly lower in the ITKi-treated group compared with the control group (**Figure 1C**).

Furthermore, in the ITKi-treated group, splenomegaly was much less severe than in the control group (**Figure 2A**). The number of total cells per spleen in ITKi-treated mice was significantly decreased on day 7 post-transplantation CD4+ T cells were specifically measured, and total CD4+ T cells per spleen were also consistent with the organ size (**Figure 2A**). CD4+ T cell subsets were then isolated and analyzed by flow cytometry. The percentages of CD4+IFNγ+ and CD4+IL-17A+ T cell subsets were significantly decreased (**Figure 2B**, **2C**). Further evaluations showed that the total cell number was proportional to the spleen size. Thus, the attenuated severity of acute rejection in ITKi-treated mice might be the cause of impaired Th1/Th17 immune responses.

Inhibition of ITK kinases impaired the CD8+ T cell function in vivo

CD8+ T cells are also considered to play an important role in the alloimmune response. Therefore, the CD8+ T cell function was assessed following the administration of ITKi in recipient mice. Levels of granzyme B and perforin in the CD8+ T cells of recipient animals assessed by flow cytometry were decreased (**Figure 3A**). Granzyme B and perforin are two important cytokines released by cytoplasmic granules in CD8+ T cells. These results may account for the favorable immunosuppressive effects of ITKi in the cardiac transplantation model.

Inhibition of ITK did not significantly affect the Th2/Treg subsets in vivo

Our results suggest that the subsets of CD4+IL-4+ (**Figure 2D**) and CD4+CD25+Foxp3+ (**Figure 3B**) T cells, as assessed in the long-term preserved grafts, were not significantly affected by ITKi. Although both T cell subsets showed a slight increase in the ITKi-treated group, this increase was not statistically significant with respect to the control group. Therefore, the prevalence of CD4+CD25+Foxp3+ and CD4+IL-4+ T
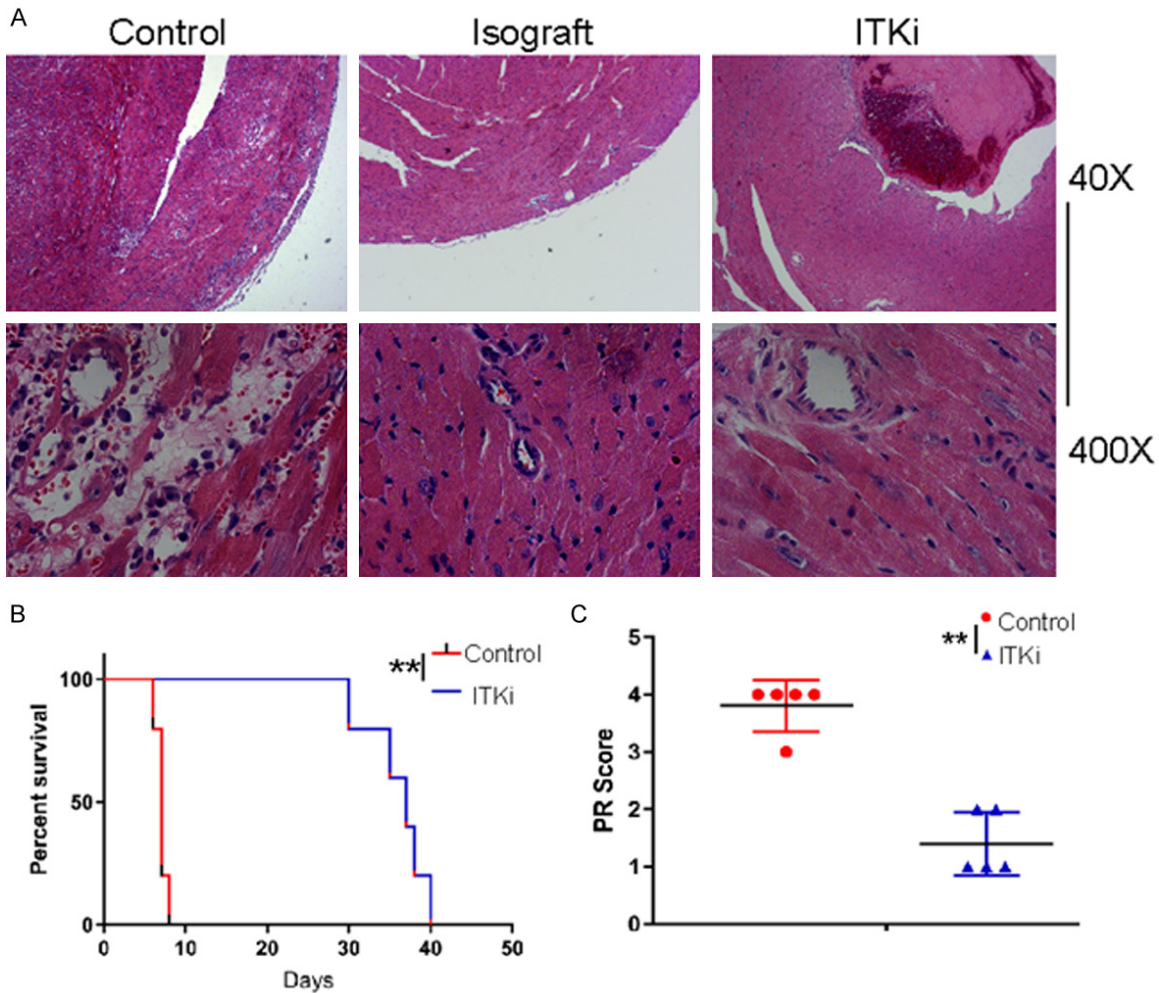
Figure 1. ITK inhibition prolongs murine cardiac allograft survival. ITKi reduced the immune rejection of allografts compared with the control group. A. H&E sections of the cardiac allografts of three groups on day 7 post-transplantation. Tissue lesion is less severe in the ITKi treated group than the control group. More pictures are seen in Figure S1. B. Survival of cardiac allografts in mice daily injected intraperitoneally with either DMSO or ITKi (20 mg/day per mouse) starting on the day of the transplant. ITK inhibition significantly prolonged murine cardiac allograft survival (36.0 ± 3.8 days vs. 7.0 ± 0.7 days). C. PR scores in the ITKi group was lower than in the DMSO group. PR scores of allograft tissue sections were evaluated on day 7 post-transplantation. Data are shown as mean ± SD for each group (n = 5).

cell subsets was not significantly affected in the cardiac transplant model under these specific conditions.

Inhibition of ITK decreased the infiltration of CD4+ T cells into the myocardial interstitium of the allografts

Infiltrated cells in the allografts were assessed by immunohistochemistry to explore the effect of ITKi on the cardiac allografts. Infiltrated CD3+ T cells and LY6G+ neutrophils in the allograft decreased 7 days after heart transplantation (**Figure 4A**, **4B**). Furthermore, infiltration of CD4+ T cells into the myocardial interstitium of

the transplanted heart was significantly reduced (**Figure 4C**). Activated CD4+ T cells that infiltrated into the allografts were critical in allo-immune responses.

Inhibition of ITK ameliorates inflammation conditions after transplantation

Expression of inflammatory factors *in vivo* contributes to allograft rejection and tissue damage, while regulation of inflammation is vital for preventing graft rejection. Inflammatory factors in the recipients' spleens and the allografts were assessed by real-time quantitative PCR. The studied factors included IFN-γ, IL-2, IL-4,
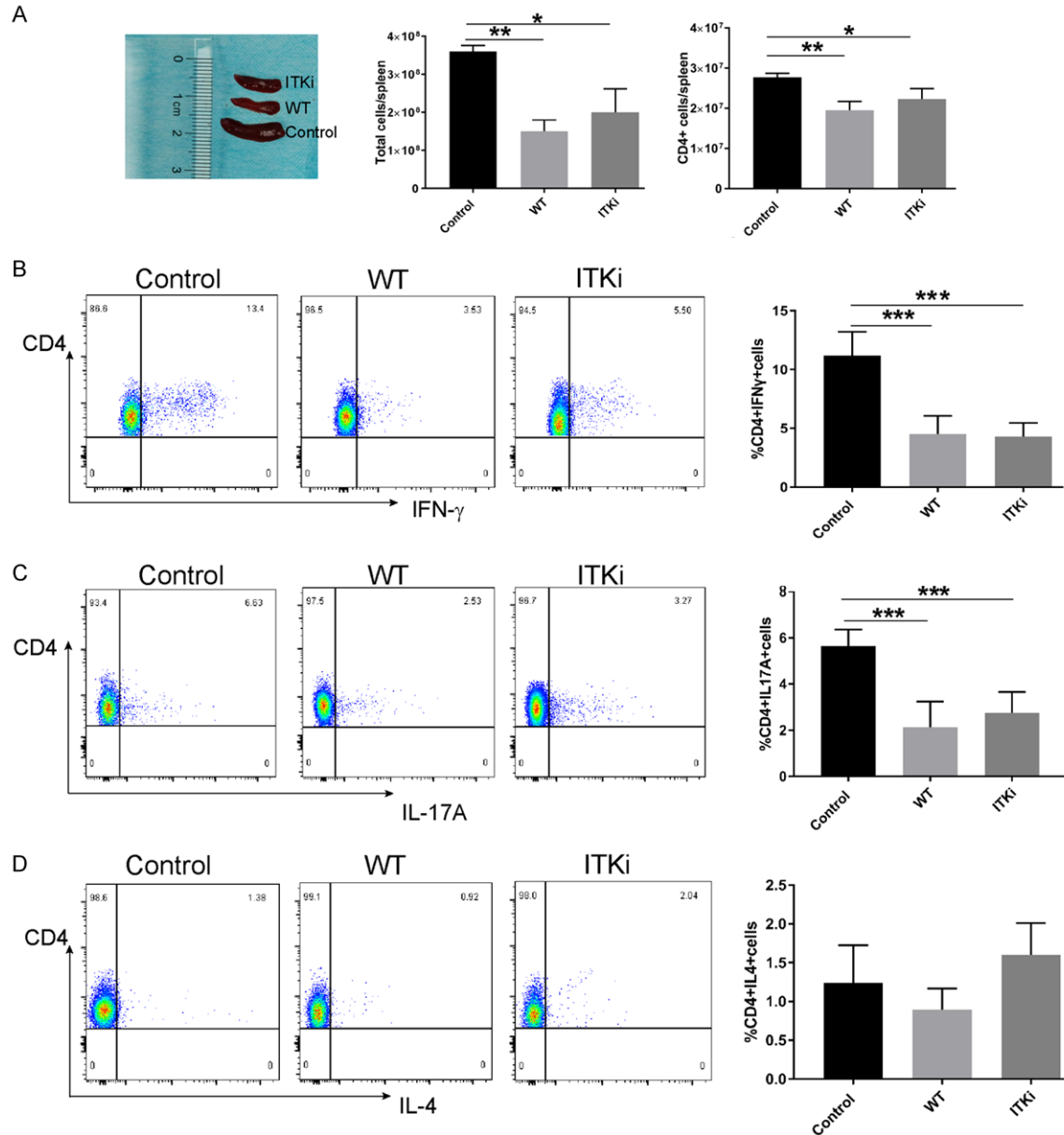
Figure 2. The CD4 subsets of lymphocytes from the recipients' spleen on day 7 were analyzed by FCM. Inhibition of ITK impaired Th1/TH17 differentiation in the cardiac transplantation model, and alleviated splenomegaly. A. Splenomegaly developed in the DMSO-treated control group but not in the ITKi-treated group. Total cells per spleen in the ITKi-treated group were less than in the control control group. Using flow cytometry, CD4$^+$ T cell numbers decreased in the ITKi-treated group compared with the control. CD4$^+$ T cell subsets were further analyzed. B. CD4$^+$IFN-γ$^+$ subsets were lower in the ITKi-treated group compared with the control group. C. CD4$^+$IL17α$^+$ subsets were lower in the ITKi-treated group compared with the control group. D. CD4$^+$IL4$^+$ subsets were not altered in the ITKi-treated group compared with the control group.

IL-17A, GM-CSF. Transcription factors included T-bet, GATA3, Rorγt, and Foxp3 (Figure 5A, 5B). We found that the pro-inflammatory factors IFN-γ and IL-2 were reduced by the ITKi treatment, indicating that ITKi may mitigate the inflammatory environment in the cardiac allografts.

Inhibition of ITK exerts effects through the PLCγ signaling pathway in vitro

The kinase ITK plays a critical role in the signal transduction downstream of the T cell antigen receptor and has been implicated in the activation of phospholipase PLC-γ1 [12]. The level of
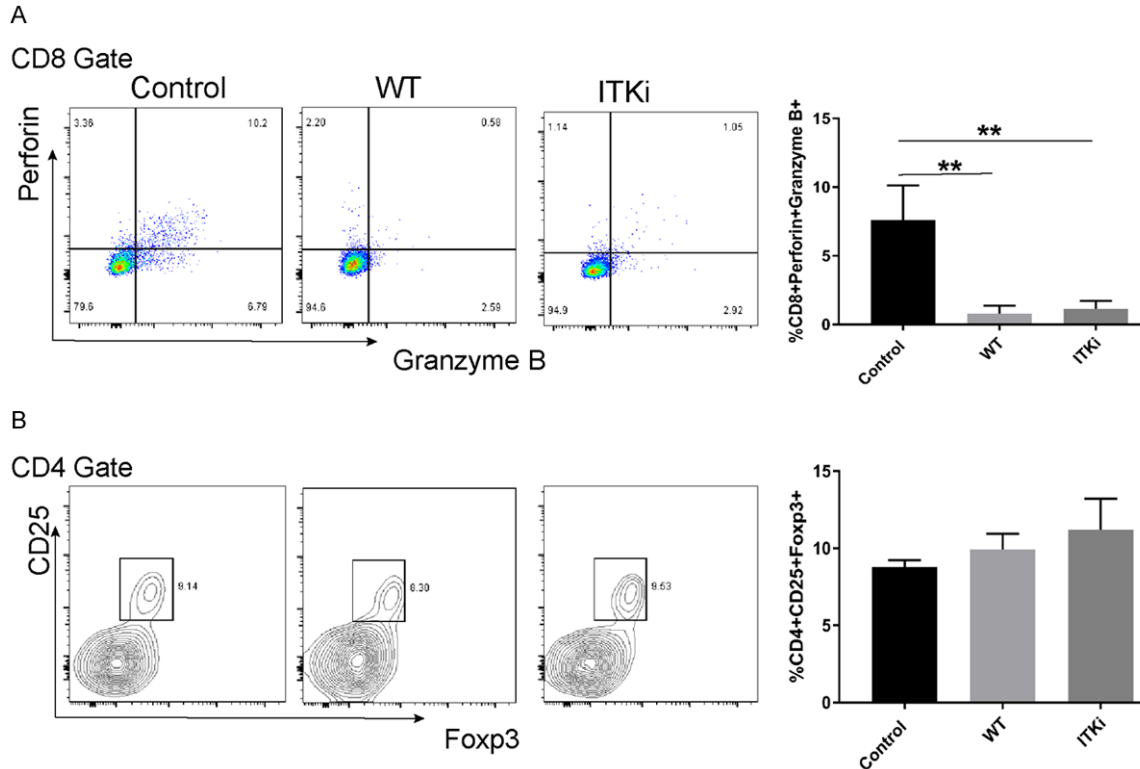
Figure 3. The CD8 T cells and Tregs from the recipients' spleen on day 7 were analyzed by FCM. CD8$^+$ function was impaired following ITK inhibition *in vivo*. A. CD8$^+$GranzymeB$^+$ and perforin$^+$ T cells were much lower in the ITKi-treated group compared with the control group. B. CD4$^+$CD25$^+$Foxp3$^+$ T cells were slightly increased (P>0.05) in the ITKi-treated group compared with the control group.

PLCγ phosphorylation was decreased in the cardiac allografts 7 days after transplantation (**Figure 5C**). To explore the underlying mechanism of the effect of ITKi on T cells *in vitro*, CD4$^+$ and CD8$^+$ cells were treated for 48 h with ITKi. Given that ITK plays its role partly through activation following TCR signaling, which requires the activation of PLCγ, the levels of PLCγ phosphorylation were investigated by immunoblot analysis. The results showed that the levels of pPLCγ were significantly decreased after the pretreatment with 0.2 μM ITKi (BMS-509744) compared with the DMSO group (**Figure 5D, 5E**). These data suggest that the alleviation of symptoms associated with severe alloimmune responses in murine models, as one of the potential benefits of ITKi, is at least partly manifested via PLCγ signaling.

Discussion

Despite the significant progress in immunosuppressive therapy, acute allograft rejection still remains a major issue occurring in about 20-40% of organ recipients and is largely mediated by T cells [13]. Allograft recipients have to rely on immunosuppressive agents to control alloreactive CD4$^+$ T cells for the rest of their life [14]. The goal for researchers and clinic physicians is to achieve longer allograft survival without any significant side effects. In our study, we explored the inhibition of ITK by its specific inhibitor BMS-509744, which was found to induce significant impairment in T cell function and a markedly prolonged cardiac allograft survival.

To test and confirm the relevance of ITKi inhibitory activity *in vivo*, we used a murine cardiac transplantation model and investigated cell-mediated immune responses in major T cell subsets, including Th1/TH2/Th17/Treg cells. We subsequently assessed the effects of ITK inhibition on CD4$^+$ T cell function *in vitro* and *in vivo*. ITKi administration ameliorated the progression of allograft rejection and the inflammatory status. Furthermore, we found that ITK
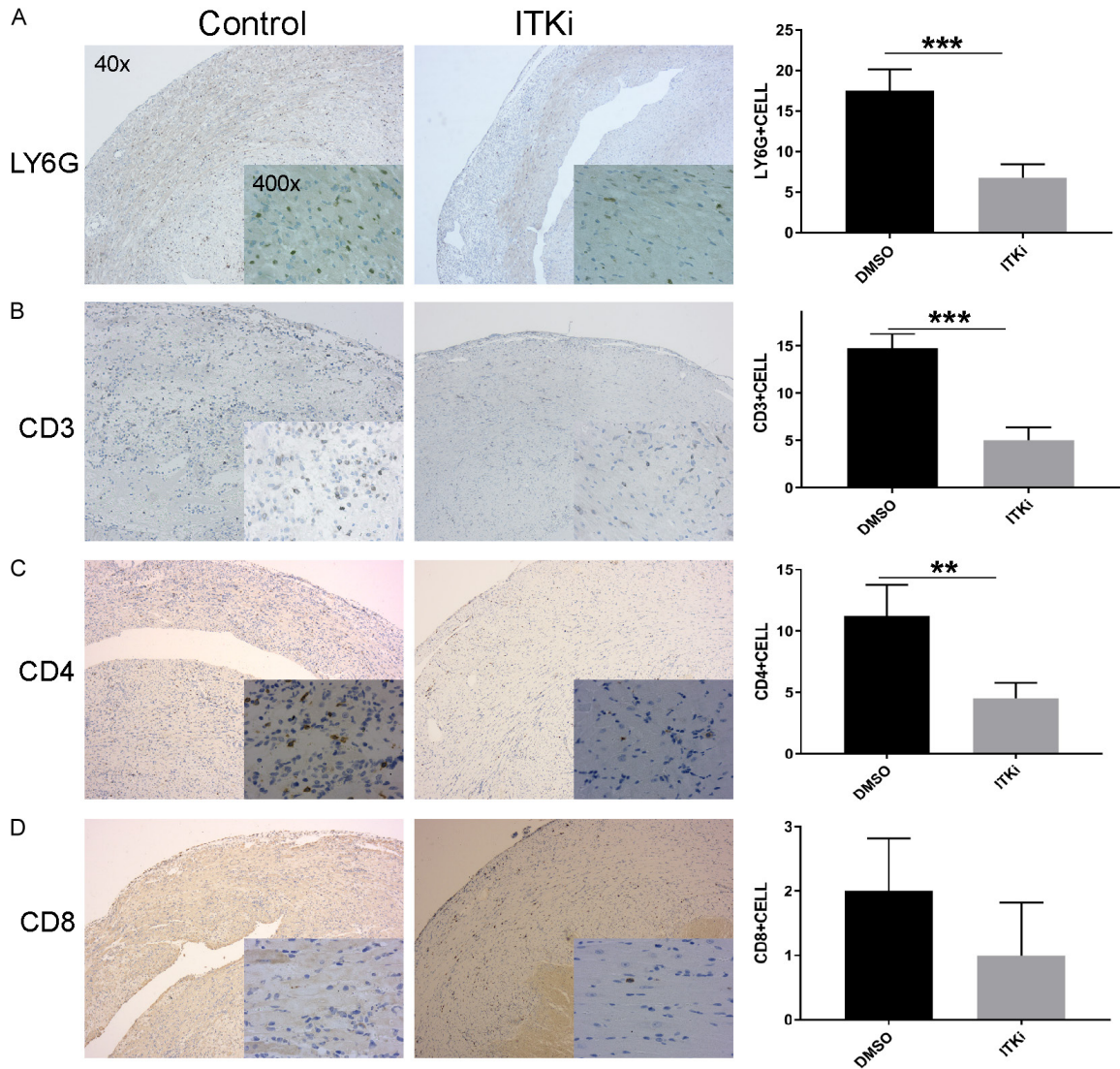
Figure 4. Infiltrated cells in the allografts were assessed by immunohistochemistry to explore the ITKi's effects on the cardiac allografts. Inhibition of ITK decreased infiltration of LY6G+, CD3+ and CD4+ T cells into the myocardial interstitium of the allografts. A. LY6G+ cells were reduced in the ITKi-treated group compared with the control group. B. CD3+ cells were reduced in the ITKi-treated group compared with the control group. C. CD4+ cells were reduced in the ITKi-treated group compared with the control group. D. CD8+ cells were not altered in the ITKi-treated group compared with the control group. Immunohistochemistry was performed on tissue sections at 7 days post-transplantation. The number of positive cells was averaged over five random high-power fields.

inhibition showed potent inhibitory effects on Th1 and Th17.

Earlier studies [15, 16] focused on the phenotype of ITK$^{-/-}$ mice, which included defects in T cell activation, differentiation, and effector function, most of which reported changes in nearly all CD4+ subsets. While consistent differences in the T cell response have been reported among the different animal models, another issue is the challenge in assessing the function of ITK in CD4+ T cells as well as in the different kinds of immune responses. Moreover, distinguishing between ITK effects and CD4+ T cell activation and differentiation due to altered T cell development is not straight-forward in ITK$^{-/-}$ mice. Our study investigated the effects of ITKi in cardiac transplantation in a more translationally relevant way.

ITK has been shown to have an essential role in TCR signaling by regulating the activation of various downstream pathways in CD4+ T cells [17]. While the relationship between ITK and
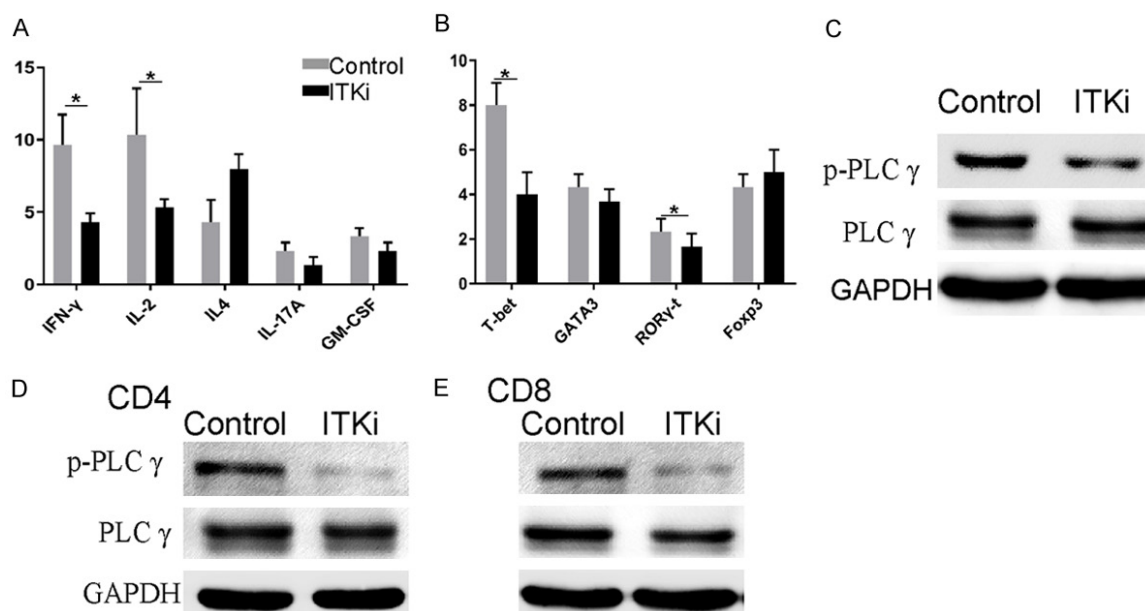
Figure 5. Inflammatory factors in the cardiac allografts were assessed.Inhibition of ITK exerts effects on PLCγ signaling pathway *in vitro.* Levels of phosphorylation of PLCγ in CD4$^+$ T cells and CD8$^+$ T were decreased by the ITKi. A, B. The levels of inflammatory factors and T-bet, GATA3, RORγt, and Foxp3, as assessed by RT-qPCR, pointed to a relatively low-inflammatory T cell phenotype. C. The levels of phosphorylation of PLCγ in the cardiac allografts were detected by immunoblot analysis, and p-PLCγ was decreased in the presence of ITKi. D. The Levels of phosphorylation of PLCγ in CD4$^+$ T cells were decreased by ITKi. E. The levels of phosphorylation of PLCγ in CD8$^+$ T cells was decreased by ITKi.

Th17 has been widely investigated [8, 18], the effects of ITK on Th1 are not well understood. Kannan reported a study on ITK$^{-/-}$ T cells in mice, which showed that ITK activity is not only required for Th2 and Th17 cell cytokine production but also for Th1 cytokine production [4]. This work provided an insight into the role of ITK signaling in the development and function of Th1. In a model of neuroinflammation, ITK$^{-/-}$ CD4$^+$ T cells showed significant reduction in the production of both Th1 and Th17 cytokines [5]. Further exploration by Lin showed that ITK inhibition reduces T-cell receptor-induced functions, including PLCγ1 tyrosine phosphorylation, calcium mobilization, IL-2 secretion, and T-cell proliferation *in vitro* in both human and mouse cells [9]. Furthermore, ITK and RLK inhibition resulted in impaired Th1 differentiation in a mouse colitis model [19] with decreased IFN-γ and IL-17A production. In our study, ITK inhibition reduced Th1/Th17 subsets after cardiac transplantation, which contributed to a prolonged allograft survival. In the case of Th2 cell differentiation, regulated by the essential transcription factor GATA3 [20], the inhibition of ITK suppressed the Th2 production and lymphocytic/neutrophilic/eosinophilic airway inflammation [20].

ITK also regulates the development and function of Treg [7]. Studies of the relationship between ITK and Treg have shown that ITK negatively regulates Treg [21, 22]. ITK$^{-/-}$ CD4$^+$ T cells were found to generate higher percentages of functional Foxp3$^+$ cells, which is associated with increased sensitivity to IL-2 [18]. ITK is a potential target for altering the balance between Treg and other CD4$^+$ subsets. In addition, ITK$^{-/-}$ CD4$^+$ T cells, preferentially developed in Treg cells *in vivo*, exhibited enhanced expression of the co-inhibitory receptor PD-1 and were suppressive in a T cell proliferation assay [22]. In agreement with most of the reports, our study showed that administration of ITKi impaired Th1/TH17, which may thus contribute to an alleviated alloimmune response.

Our study also showed an impaired CD8$^+$ functions with administration of ITKi, which may explain the observed immunosuppressive effects in the cardiac transplantation model. CD8$^+$ T cells showed reduced secretion of granzyme B and perforin upon ITKi treatment. This is in agreement with an earlier study that revealed that ITK regulates the quality of the antigen-specific CD8$^+$ T cell cytokine response *in vitro* [23]. The support of CD4$^+$ T cells to achieve

CD8^{+} activation was vital, as CD4^{+} T cell support is essential for CD8^{+} CTL priming [24]. This indicates that ITKi impaired both CD4^{+} and CD8^{+} T cells in our research, and impaired CD4^{+} cells were less potent for CD8^{+} CTL activation. However, in a patient with a homozygous missense mutation in the kinase domain of ITK that is prone to EBV-positive lymphomatoid granulomatosis, the stimulation of T cells resulted in reduced phosphorylation of ITK, PLCγ, and PKC, while the cytotoxicity and degranulation of CD8^{+} T cells was impaired [25]. This indicates that ITK inhibition induced a more friendly environment to allografts by impairing both CD4^{+} and CD8^{+} functions.

Activation of PLC-γ is a critical step in T cell antigen receptor signaling, which subsequently leads to an increased calcium flux. To further explores the effects of ITKi on T cells, CD4^{+} and CD8^{+} subsets were freshly isolated via magnetic sorting from spleens. *In vitro* phosphorylated PLCγ1 was reduced after blockade of ITKi in both CD4^{+} and CD8^{+} cells. Moreover, negative regulation of ITK has been implicated in the regulation of calcium mobilization and extracellular signal-regulated activities [12]. The involvement of phosphatases in the TCR-signaling cascade is also important for the development of T cells [26]. Previous studies using primary murine ITK^{-/-} T cells demonstrated the requirement of the kinase for robust T cell activation in response to TCR with co-stimulatory receptor signaling [15]. Moreover, ITK inhibition was reported to be efficacious in mouse models of graft-versus-host disease, experimental autoimmune encephalomyelitis (EAE), and colitis [5, 27].

ITKi has also been reported to ameliorate allergic responses [6]. Cells isolated from ITK^{-/-} mice or ITKi-treated mice show defects in degranulation and cytokine secretion responses downstream of TCR signaling. Lin et al. showed that T cell proliferation and expansion *in vitro* can be induced by irradiated APC and subsequently suppressed by BMS-509744 [9]. BMS-509744 was not cytotoxic to mouse lymphocytes and human PBMC cells, as measured by a lactate dehydrogenase release assay [10]. It should be noted that this study has solely examined the alloimmune circumstance, with a special focus on immunosuppressive effects. In contrast, our study revealed for the first time its potential in alleviating Th1/Th17 alloimmune responses in

a mouse model of cardiac transplantation. Despite some disparities between studies, in general, our results have provided solid evidence of the vital role of ITKi in regulating differentiation and activation of CD4^{+} T cells.

Taken together, the data presented here indicates that the selective, small-molecule ITK inhibitor BMS-509744 is effective in maintaining alloimmune immunosuppression. Our experiments indicate that ITKi alleviates the allograft rejection mainly by impaired PLCγ phosphorylation of CD4^{+} T cells in alloimmune responses in the murine cardiac transplantation model. Our results support the potentiality of ITKi as an alternative strategy to alleviate allograft immune rejection.

Disclosure of conflict of interest

None.

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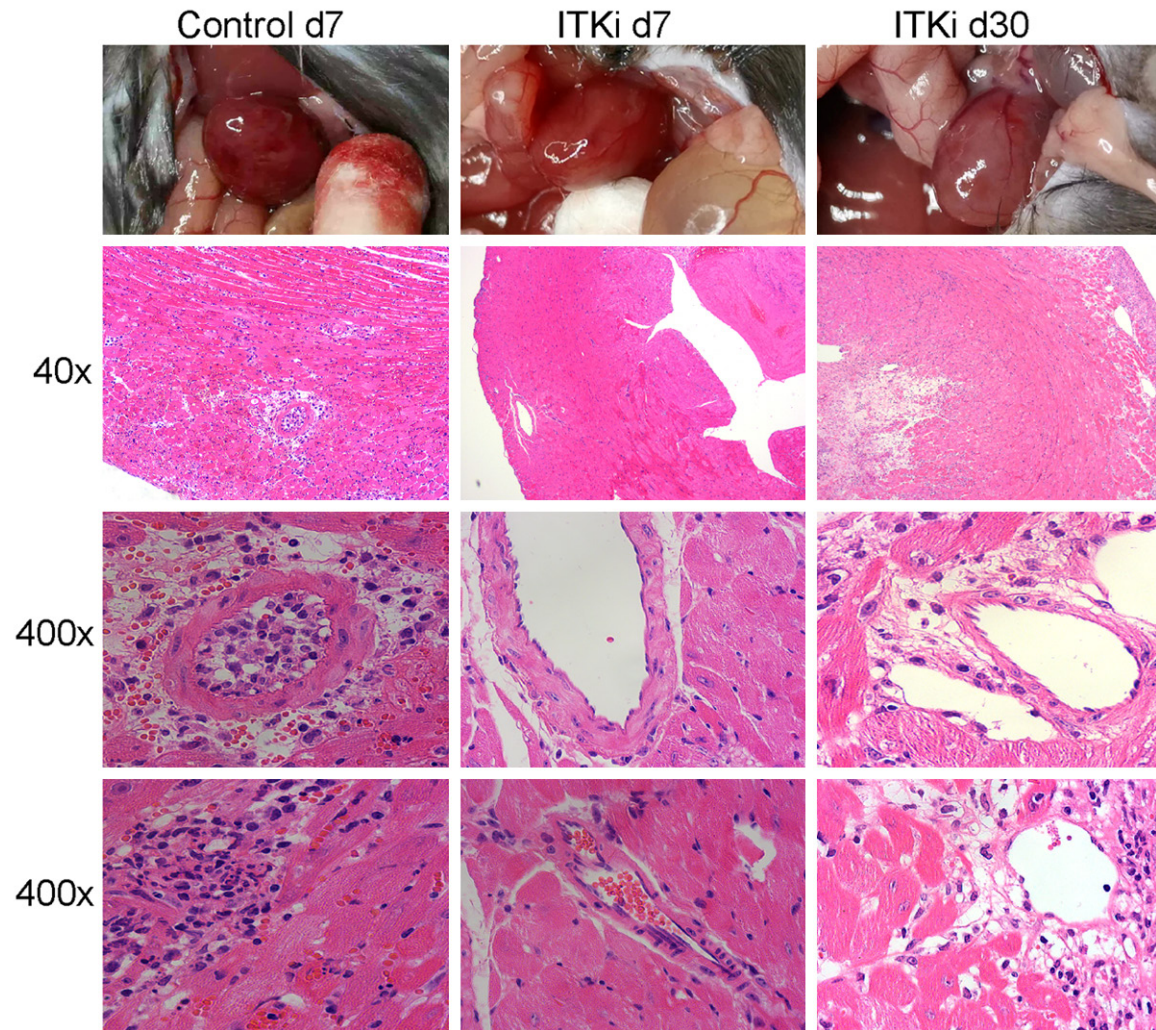
Figure S1. ITK inhibition prolongs murine cardiac allograft surviva. Acute rejection was alleviated by ITK inhibition. From left to right, H&E sections of the cardiac allografts on day 7 in the control group. H&E sections of the cardiac allografts on day 7 in the ITKi group. H&E sections of the cardiac allografts on day 30 in the ITKi group.