Ablation of Survivin in T Cells Attenuates Acute Allograft Rejection after Murine Heterotopic Heart Transplantation by Inducing Apoptosis

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Although studies in oncology have well explored the pharmacological effects of Birc5, little is known about its role in allogeneic T-cell responses. Therefore, the present study used a mouse model of acute heart allograft rejection to investigate the protective effect and mechanism of conditional knockout of Birc5 in T cells. Survivin (encoded by Birc5) was upregulated in T cells activated in vivo and in vitro. Deletion of Birc5 in T cells attenuated acute heart allograft rejection by reducing the ratio of effector to naive T cells and Th1 to Tregs. In addition, deletion of Birc5 had no noticeable effect on proliferation but on apoptosis and the secretion of IFN-γ. The results revealed a significant increase in the percentage of Annexin V positive CD4+ T cells in the Birc5−/− group, compared to the WT. Moreover, there was significant increase in early apoptotic alloreactive T cells in Birc5−/− mice and this was partly mediated by caspase-3. Furthermore, treatment with YM155 inhibited acute heart allograft rejection in vivo and increased T-cell apoptosis in healthy human PBMCs in vitro. The results highlight a potential therapeutic target for the prevention and treatment of acute transplant rejection.

Keywords: transplantation immunology, survivin, Birc5, T cell, apoptosis

INTRODUCTION

Heart transplantation is the most effective and essential clinical treatment for terminal refractory heart failure caused by various diseases (1–3). Although great progress has been made on controlling acute transplant rejection, the long term survival of patients is affected by such factors as the rejection-mediated graft vascular disease (which causes graft ischemia and
functional failure) and the side effects caused by various immunosuppressive agents such as infection, renal fibrosis and malignant tumors (4, 5). Therefore, it is necessary to identify new targets in order to control transplant rejection or enhance immune tolerance. Given that T cells were proven to play an indispensable role in the occurrence of rejection after transplantation (6, 7), T cell survival and function has gained popularity in the field of transplantation immunology.

In addition, Birc5 encodes the survivin protein which is a known inhibitor of apoptosis (8). Notably, survivin plays an essential role in mitosis by ensuring proper separation of chromosomes (9, 10) and also prevents apoptosis by hindering the activation of the caspase pathway (11). Moreover, survivin is used as a biomarker of resistance to chemotherapy, increased metastatic activity and risk of tumor recurrence, in cancer therapy (12). Survivin also plays an essential role in the maturation and proliferation of T cells (13) and in regulating T cell responses (14, 15). Additionally, upregulated levels of survivin was shown to affect the viability and responses of infected CD4+ T cells, in antiviral immunity (14). Furthermore, silencing the Birc5 gene leads to enhanced apoptosis as well as reduced viability and proliferation of inflammatory T cells in autoimmune diseases (16, 17). Nonetheless, little is known about the role of survivin in transplantation immune responses.

Consequently, the present study demonstrated that deletion of Birc5 in T cells attenuates acute heart allograft rejection, which is associated with decreased alloreactive T cell responses and enhanced T cell apoptosis in a caspase-3-dependent manner. Moreover, a mouse model was used to show that treatment with inhibitor, YM155 can also attenuate acute heart allograft rejection. The results therefore showed that survivin may be a new drug target for use in the treatment of acute transplant rejection.

MATERIALS AND METHODS

**Animals**

The B6.129P2-Birc5tm1Mak/J (Birc5flax/flax) and CD4-Cre mice were obtained from the Jackson Laboratories (Bar Harbor, ME) while the C57BL/6 and BALB/c mice were purchased from the Shanghai Model Organisms Center (Shanghai, China). Thereafter, the Birc5flax/flax mice were crossed with the CD4-Cre mice to generate the Birc5flax/flax CD4-Cre mice (Birc5−/−). All the mice were bred under specific pathogen-free conditions in the Laboratory Animal Center, Huazhong University of Science and Technology (Wuhan, China). Moreover, male mice that were 8 to 10 weeks old were used in the subsequent experiments. All the animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College (Wuhan, China).

**The Heterotopic Heart Transplantation Model**

The heterotopic cardiac transplantation model was generated as previously reported (18) and allograft function was monitored every day through palpation. Briefly, hearts were obtained from BALB/C mice then stitched to the aorta abdominals and postcava of C57BL/6 mice. The mice were divided into three groups, i.e., the isograft group and 2 allograft categories. The isograft group received the C57BL/6 heart then treated with saline while the other two allograft categories received intraperitoneal (i.p.) injection of either saline or YM155 (5 mg/kg) on day 1, 3 and 5 after the operation. Additionally, the spleen or grafts from recipient mice were extracted on the 6th day for analysis.

**Isolation of Heart-Infiltrating CD45 Cells**

The heart-infiltrating cells were isolated, as previously described (18). Briefly, the heart tissues were obtained and cut into pieces after which the tissue fragments were digested with 1 mg/mL of collagenase B (Roche 11088815001) or Collagenase Type 2 (Sangon Biotech A004174-0100) in HEPES buffer then rotated gently for 45 min at 37°C. In addition, cell suspensions were filtered with 70-µm cell strainers (BIOFIL) and Percoll (Beijing Solarbio Science & Technology Co., Ltd.) was used to purify heart mononuclear cells through density centrifugation.

**In Vitro T Cell Sorting, Activation, and the CTV Labelled Proliferative Assay**

Naive CD4+ T cells were isolated from WT B6, Birc5flax/flax CD4-Cre or Birc5flox/flox mice through magnetic bead sorting of CD3+CD4+CD25+ T cells using the LS columns (Miltenyi). Purified CD4+ T cells (2x10^5 cells/well) were then plated in 96-well plates that had been pre-coated with 5 µg/mL of anti-CD3 Ab (BioLegend) for 4 hours, after which soluble anti-CD28 Ab (BioLegend) was added (final concentration, 1 µg/mL). Afterwards, T cells were cultured and treated as described in each experiment then finally collected for analysis. In order to evaluate the effect of Birc5 knockout on the proliferative potential of CD4 T cells in vitro, the study labelled the cells using the CellTrace Violet (CTV) fluorochrome then stimulated them in 96-well plates coated with the anti-CD3 Ab and soluble anti-CD28 Ab. The rest of the experiments including Mixed-lymphocyte Reactions (MLR) (19), western blotting (20), FCM and ELISA (21) are included in the supplemental experimental procedures.

**RESULTS**

**Survivin Was Upregulated in Allograft-Infiltrated T Cells During Acute Allograft Rejection**

Based on the procedure in Figure 1A, the study separated the infiltrated CD45+ cells on the 6th day after heart transplantation then assessed the levels of survivin in alloreactive T cells. The FCM histogram plots demonstrated that survivin was upregulated in alloreactive CD4+ and CD8+ T cells (Figures 1B–E), in the heart. Additionally, the study used live/dead staining to gate out the dead cells and in the next gate the Annexin V positive cells were considered as early apoptotic cells. Therefore, flow
cyclotherapy was used to examine the levels of early apoptotic alloreactive T cells. Interestingly, the results showed that the percentage of Annexin V positive alloreactive T cells was higher than that in the isograft group (Figures 1F, G).

**Conditional Knockout Birc5 in T Cells Alleviates Acute Allograft Rejection Following Murine Heterotopic Heart Transplantation**

In order to verify the effect of survivin on the rejection of cardiac transplants, BALB/c hearts were transplanted into Birc5<sup>lox/lox</sup>CD4<sup>-Cre</sup><sup>+</sup> (Birc5<sup>+/+</sup>) or Birc5<sup>lox/lox</sup>CD4<sup>-Cre</sup> (WT) mice. The results showed that the Mean Survival Time (MST) of the grafts was longer in Birc5<sup>+/−</sup> recipients than in the control group (Figure 2A). Additionally, H&E staining of the heart grafts on the 6th day after transplantation showed that mice in the Birc5<sup>+/−</sup> group had lower heart inflammatory cell infiltration scores compared to the control category (Figures 2B, C). Moreover, the spleen of mice in the Birc5<sup>+/−</sup> group had no evident splenomegaly. After grinding, filtration and red blood cell lysis using ACK lysing buffer, we calculated the cell number in the spleen. There was also a remarkable decrease in the number of spleen cells of mice in the Birc5<sup>+/−</sup> group compared to those in the control category (Figures 2D, E). The percentage and absolute cell numbers of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in spleen from indicated groups was shown in Supplemental Figures 3D, E.

**The Effect Knocking Out Survivin on the Subsets of T Cells in the Spleen After Heart Transplantation**

The study then analyzed the effects of Birc5 knockout on T-cell subpopulations in the spleen after heart transplantation. Therefore, the spleen was harvested on the 6<sup>th</sup> day after transplantation then assessed through flow cytometry. The gating strategy in the spleen is shown in Supplemental Figure 3A as previous studies (22–24). The findings revealed a significant decrease in the proportion of effector/ effector memory (CD62L<sup>−</sup>CD44<sup>+</sup>) T cells in the Birc5<sup>+/−</sup> group. Meanwhile, the proportion of naive (CD62L<sup>+</sup>CD44<sup>−</sup>) T cells was increased (Figures 3A, B). The CD4<sup>+</sup> T graph is shown in Supplemental Figures 3B, C. In addition, the proportion of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells significantly increased in Birc5<sup>+/−</sup> recipients although there was a decrease in CD8<sup>+</sup>IFN-γ<sup>+</sup> and CD4<sup>+</sup>IFN-γ<sup>+</sup> T cells (Figures 3C–H). The percentage of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in spleen from indicated groups was shown in Supplemental Figure 3D. The absolute cell numbers relative was shown in Supplemental Figures 3E, F. Moreover, the flow cytometry plots showed that the spleen of Birc5<sup>+/−</sup> mice had a higher proportion of Annexin V<sup>+</sup> apoptotic CD4<sup>+</sup> T cells than the WT group (Figures 3I, J).

**Knock-Out of Birc5 INDUCED MORE APOPTOSIS of T Cells and Had No Obvious Effect on Proliferation**

The study then cultured the same number of CD4<sup>+</sup> T cells from both Birc5<sup>+/−</sup> and the WT mice, with or without activation. 48 hours later, the number of CD4<sup>+</sup> T cells in Birc5<sup>+/−</sup> mice was less than that in the control group (Figure 4A). The western blotting demonstrated that survivin was upregulated in T cells after activation (Supplemental Figures 2A, B). Knock-out of Birc5 could down-regulate the survivin expression (Supplemental Figures 2C, D). In addition, the study examined whether the
reduced number of CD4+ T cells was due to decrease in proliferation or apoptosis. The results revealed no significant differences in the levels of the proliferation marker, Ki67 between the two groups (Figures 4B, C). Moreover, naive CD4+ T cells were labelled with CTV then the proliferative assay was conducted. The findings showed similar levels of the proliferation in CD4+ T cells following the dilution of CTV (Figure 4D, E). On the contrary, the Birc5-/- mice had a higher percentage of Annexin V+ apoptotic T cells (Figure 4F, G).

The Apoptosis Induced by Birc5 Knockout in T Cells Was Partly Dependent on Caspase3 Activation

Thereafter, the study cultured the same number of CD4+ T cells from Birc5-/- and WT mice for 48 hours, in order to examine the underlying mechanism of apoptosis induced by Birc5 knockout. The results showed that the number of CD4+ T cells from Birc5-/- mice was less than that in the control group. However, the number increased after treatment with the Caspase 3 inhibitor, Ac-DEVD-CHO (Figure 5A). The study then assessed whether the decrease in the number of CD4+ T cells was due to proliferation or apoptosis. Analysis of the proliferation marker, Ki67 revealed no significant difference between the two groups (Figure 5B, C). However, the proportion of Annexin V+ apoptotic T cells was higher in Birc5-/- mice than in the controls. Additionally, treatment with Ac-DEVDO-CHO led to a decrease in the proportion of apoptotic cells (Figures 5D, E). These results therefore suggested that Ac-DEVDO-CHO restored the number of cells and decreased the percentage of apoptotic cells without affecting the ratio of ki67+ cells. Furthermore, the levels of Cleaved-CASP3 and Cleaved-PARP1 were assessed in the stimulated CD4+ T cells. The results revealed significant upregulation of Cleaved-CASP3 and Cleaved-PARP1 in survivin deficient CD4+ T cells, compared to the controls. However, treatment with Ac-DEVDO-CHO restored the levels of both Cleaved-CASP3 and Cleaved-PARP1 (Figures 5F, G).

YM155 Suppresses Acute Allograft Rejection Following Murine Heterotopic Heart Transplantation

YM155 is a small molecule that is known to inhibit survivin. Notably, YM155 can selectively inhibit the expression of survivin at both the mRNA and protein levels. YM155 was also shown to have anticancer activity in many malignancies (25). In this study,
survivin was up-regulated when T cells were in the activation stage but was down-regulated after treatment with YM155 (Supplemental Figures 2E, F). In addition, YM155 increased the apoptosis of T cells in vitro although treatment with Ac-DEVD-CHO reversed this effect (Supplemental Figures 1A, B). Thereafter, the study choose a safe and effective dose of YM155 as reported in previous research, for animal experiments (26, 27). Mice in the isograft group were treated with the vehicle (PBS) while those in the allograft group received either YM155 (5mg/kg on -1, 1, 3, 5 days) or the vehicle through intraperitoneal injection. The Mean Survival Time (MST) of grafts in mice treated with YM155 was 14 days which was 8 days longer than that in the vehicle group (n = 5; p < 0.01; Figure 6A). Additionally, there was a significant decrease in the number of splenocytes in mice treated with YM155, on the 6th day (Figure 6B). Moreover, H&E staining of the grafts showed that the YM155 group had cell infiltration scores lower than the vehicle category (2.40 ± 0.54 vs. 1.00 ± 0.70; Figures 6C, D).
YM155 Reduces the Secretion of IFN-γ in a Human Mixed Lymphocyte Reaction

Similarly, YM155 increased the apoptosis of human PBMC T cells in vitro (Figure 6E). In order to evaluate the effect of YM155 on the secretion of inflammatory factors from T cells, human MLR was conducted and the supernatant was collected on the 5th day for ELISA. The results showed that the concentration of IFN-γ was significantly lower in the YM155 group than in the vehicle category (Figure 6F).

DISCUSSION

The present study used a mouse model of acute heart allograft rejection to demonstrate the protective effect of T-cell specific Birc5 knockout and its underlying mechanism. In addition, the study used an animal model to explore the potential benefit of targeting survivin with YM155 in reducing acute allograft rejection. Previous studies showed that survivin regulates genes associated with the proliferation and development of hematopoietic stem cells and thymic T cells (13, 28). Additionally, survivin was reported to facilitate lymphocyte proliferation and promoted the maturation of T cells in arthritis (17). Given that Birc5 has been shown to affect T-cell responses and that it increases during alloimmune responses, the present study further explored the role of Birc5 in graft rejection.

Notably, Andersson et al. reduced the levels of survivin in vivo using a lentivirus shRNA and observed a significant increase in the levels of Tregs. Their results demonstrated that the more survivin was inhibited, the more Tregs were generated (17). Additionally, YM155 (A small molecule that inhibits survivin) may be used to inhibit survivin in vivo and in vitro experiments. Notably, the antitumor activity of YM155 was first reported in a human malignant melanoma model and the
molecule can also be applied in immune-related diseases (29). It was also reported that drugs could attenuate acute heart rejection by antagonizing the activity of key transcription factors that determine the differentiation of T cells subgroups (30). However, it is still unclear whether survivin limits the differentiation of alloreactive T cells to Tregs in an inflammation environment or only changes the flexibility of mature Tregs, leading to allotransplant rejection. The mechanisms underlying the effect of survivin are also unclear and should therefore be explored further.

In addition, previous studies showed that reduced the levels of survivin led to a decrease in the population of effector T cells in the spleen and local infiltration of T cells (17). Similarly, the present study showed that there was a significant decrease in the number of effector effector memory T cells (CD62L−CD44+) and a decrease in the levels of IFN-γ in T cells in YM155 group, suggesting that a decrease in the number of effector memory T cells is associated with an increase in the number of Tregs.

Moreover, a previous study showed that reducing the levels of survivin with YM155 in human adult T-cell lymphoma cells
affected cell proliferation and induced cell death (31). Additionally, it was reported in HIV-1 patients, that resting CD4+ T cells with higher levels of \textit{Birc5} mRNA had a long term proliferative ability while those treated with YM155 underwent more apoptosis and cell death (14). Furthermore, previous studies showed that \textit{Birc5}−/− mice had impaired T cell proliferation and a reduced number but still with basically naturally phenotypes, consistent with the results obtained in the present study (13). In addition, the results herein revealed that knock out of \textit{Birc5} suppressed the production of IFN-γ in T cells, similar to the results obtained from previous research (17).

The study also selected a safe dose of YM155 for \textit{in vivo} and \textit{in vitro} experiments, based on previous research (26, 32). The drug was administered through the intraperitoneal route and therefore not only affected T cells but also other cell population. According to previous research, over expression of survivin in grafts may suppress inflammation resulting from ischemia/reperfusion injury (33). However, the effects of survivin on host T cells are yet to be elucidated. Notably, survivin may have distinct functions in different cells, at various stages and even in different subcellular localizations. In cardiomyocytes for example, it inhibits apoptosis by reducing the expression of active Caspase-3, leading to less damage in the inception phase (34). In addition, previous research revealed that survivin inhibits apoptosis by maintaining mitochondrial integrity and associating with many molecules in the mitochondria (35). Up-regulation of survivin was also shown to confer protection from anthracycline-induced cardiotoxicity (36). Additionally, inhibition of caspases may have an effect on the microvascular endothelial cells in allografts, subsequently alleviating cardiac rejection (37). In the present study, the results showed that the YM155-induced cell apoptosis \textit{in vitro} was partly mediated by activation of caspases and could be reversed upon the addition of a specific Caspase 3 Inhibitor. Moreover, PBMCs from healthy humans were treated with YM155 and similar results were obtained. The findings also showed that targeting survivin with YM155 could reduce the secretion of IFN-γ in human T lymphocytes.

In summary, the present study reported for the first time the effect of \textit{Birc5} knockout on the survival of cardiac allografts. The results suggested that regulation of the caspase pathway may be partially responsible for the immune alterations that prolonged graft survival. Moreover, interfering with the expression of survivin
using YM155 may be an effective strategy to inhibit rejection after transplantation.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

**AUTHOR CONTRIBUTIONS**

HX, JY, JX and JW conceived and designed the study. HX and JY contributed to the writing of the manuscript. JC, and LJ: feeding cage and identification mice. HX, JY, YL, and JC analyzed results and discussed the results. HX, JY and JW coordinated this work, and all authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.710904/full#supplementary-material

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