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T follicular helper and memory cell responses and the mTOR pathway in murine heart transplantation

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Abstract

BACKGROUND: Mammalian target of rapamycin (mTOR) inhibitors are valuable immunosuppressants in clinical transplantation, but mTOR regulation of allogeneic T cell responses is not fully understood. Here, we investigated the effects of T cell-specific mTOR deletion on allogeneic T cell responses and heart transplant survival.

METHODS: Wild type C57BL/6, *Mtor*^{fl/fl} *Cd4-Cre*, *Stat3*^{fl/fl} *Cd4-Cre*, and *Mtor*^{fl/fl} *Stat3*^{fl/fl} *Cd4-Cre* mice were transplanted with BALB/c heart allografts, with or without BALB/c skin-sensitization. Graft survival and histology, as well as T cell frequencies and phenotypes, were evaluated after transplantation.

RESULTS: In the absence of donor skin-sensitization, long-term heart allograft survival was achieved in *Mtor*^{fl/fl} *Cd4-Cre* recipients, which was associated with significantly decreased frequencies of CD62L⁺CD44⁺ effector T cells and BCL-6⁺CXCR5⁺ T follicular helper (Tfh) cells in the periphery. Long-term heart allograft survival was also achieved in donor skin-sensitized *Mtor*^{fl/fl} *Stat3*^{fl/fl} *Cd4-Cre* mice, whereas heart allograft survival was prolonged in donor skin-sensitized *Mtor*^{fl/fl} *Cd4-Cre* and *Stat3*^{fl/fl} *Cd4-Cre* mice.

CONCLUSIONS: mTOR is required for Tfh cell response in murine heart transplantation. T cell-specific deletion of both mTOR and Stat3 abrogates memory response to heart transplants. These findings help us to better understand the molecular mechanisms underlying T cell immunity to transplanted organs.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose.

INTRODUCTION

Organ transplantation is a life-saving procedure for patients with end-stage organ failure, but long-term transplant survival is limited by immune rejection and the adverse effects of immunosuppressive drugs. CD4⁺ T helper (Th) cells are central to orchestrating immune responses against transplant organs (1, 2). Th1 cells induce transplant damage directly through the Fas-Fas ligand, or indirectly by promoting cytotoxic CD8⁺ T cell activity and macrophage-mediated delayed type hypersensitivity (3). In mice with deletion of the Th1 master regulator T-bet, Th17 cells become the critical mediators of transplant rejection (4). T follicular helper (Tfh) cells also contribute to allograft rejection by driving alloantibody responses (5), but the molecular mechanisms underlying allogeneic Tfh cell responses remains unclear.

Memory T cells exert significant impact on transplant rejection and tolerance (6). Allogeneic memory T cells are not only generated following alloantigen sensitization, such as via blood transfusion and previous transplantation, but they can also be generated through homeostatic cell proliferation and heterologous immunity (7, 8). It is commonly recognized that memory T cell-mediated rejection is extremely difficult to prevent. Approaches which induce transplant tolerance in naïve animals often fail to do so in animals enriched with allogeneic memory T cells. For instance, while costimulatory blockade is effective in inducing transplant tolerance in mice, it fails to prevent rejection in donor alloantigen pre-sensitized mice (9–11). Thus, for more effective prevention of transplant rejection, it is essential to define the key regulators that drive memory T cell responses.

The mammalian target of rapamycin (mTOR) controls multiple aspects of the T cell response (12). For instance, mTOR promotes the differentiation and function of multiple Th cell subsets, such as Th1, Th2, Th17, and Tfh cells (13, 14). In contrast, mTOR suppresses the differentiation of memory CD8⁺ T cells following viral infection and vaccination (15). It remains unclear whether mTOR also exerts these opposing effects on Th and memory cell responses in the context of transplantation. mTOR inhibitors sirolimus and everolimus are used as immunosuppressants after organ transplantation, demanding further clarification of mTOR biology in allogeneic T cell responses.

We investigate the critical regulators in allogeneic T cell responses by using the *Cd4-Cre* system to delete the floxed genes of interest in T cells (16, 17). Herein *Mtor^{fl/fl}Cd4-Cre* mice were generated to study the effects of T cell-specific mTOR deletion on allogeneic T cell responses and heart transplant survival. Long-term heart allograft survival was achieved in *Mtor^{fl/fl}Cd4-Cre* recipient mice, which was associated with significantly decreased frequencies of CD62L⁺CD44⁺ effector T cells and BCL-6⁺CXCR5⁺ Tfh cells in the periphery. In donor skin-sensitized *Mtor^{fl/fl}Cd4-Cre* recipients, heart allograft survival was also significantly prolonged. Moreover, long-term heart allograft survival was successfully achieved in donor skin-sensitized *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* recipients, in which both mTOR and Stat3 were specifically deleted in T cells. Hence, mTOR promotes both primary and memory T cell responses in the transplantation setting.

MATERIALS AND METHODS

Mice

C57BL/6 (B6), BALB/c, *Cd4-Cre*, *Mtor^{fl/fl}*, and *Stat3^{fl/fl}* mice were purchased from Jackson Laboratory (Bar Harbor, MA). A further description of these mouse strains is listed in Supplementary Table S1. *Mtor^{fl/fl}* and *Stat3^{fl/fl}* mice were crossed with *Cd4-Cre* mice to create *Mtor^{fl/fl}Cd4-Cre*, *Stat3^{fl/fl}Cd4-Cre*, and *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* mice. Mice were housed in a specific pathogen free facility at Houston Methodist Research Institute in Houston, Texas. All animal experiments in this study were approved by the Houston Methodist Animal Care Committee in accordance with institutional animal care and use guidelines.

Murine skin and heterotopic heart transplantations

Hearts from BALB/c donors were transplanted into *Mtor^{fl/fl}Cd4-Cre*, *Stat3^{fl/fl}Cd4-Cre*, *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre*, and B6 mice by a previously described method (17). Heart graft survival was monitored daily by palpation, and the day of complete heartbeat cessation was considered as the day of rejection. BALB/c ear skin allografts were transplanted onto *Mtor^{fl/fl}Cd4-Cre*, *Stat3^{fl/fl}Cd4-Cre*, *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre*, and B6 mice by a previously described method (16). More than 80% necrosis of the donor skin tissue was considered as rejection. At more than 30 days after skin transplant rejection, recipients were transplanted again with BALB/c heart allografts to investigate allogeneic memory responses.

Histology and immunofluorescence staining

Hematoxylin & eosin (H&E) staining was performed on paraffin sections of heart allografts. Immunofluorescence staining of CD3 (clone 145–2C11; eBioscience) and DAPI (eBioscience) was performed on frozen sections of heart allografts. Immunofluorescence staining of PNA (Vector laboratories), IgM (ab190369, AbCam), and CD4 (ab25475, AbCam) was performed on frozen sections of spleens. Staining method has been previously described (18).

Flow cytometry

Splenocytes and cultured T cells were analyzed on a LSR II flow cytometer (Beckton Dickinson), and the resulting data was processed by using FlowJo v10 software (Tree Star, Inc.). Fluorochrome-conjugated antibodies (BioLegend or Thermo Fisher Scientific) used for flow cytometry are as follows: specific for mouse CD4 (clone GK1.5), CD8a (53–6.7), GL7 (GL7), CD62L (MEL-14), CD44 (IM7), PD-1 (29F.1A12), BCL-6 (K112–91), B220 (RA3–6B2), and CXCR5 (2G8). Dead cells were excluded from the analysis by using the Zombie Aqua™ Fixable Viability Kit (BioLegend). T cell proliferation was assessed by using the CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher Scientific).

In vitro T cell stimulation and proliferation

Naive (CD62L⁺CD44[−]) CD4⁺ and CD8⁺ T cells were sorted from the splenocytes of B6 and *Mtor^{fl/fl}Cd4-Cre* mice by a FACSaria flow cytometer, and activated by 4 µg/ml plated-bound anti-CD3 (145–2C11; BioLegend) and 2 µg/ml soluble anti-CD28 (37.51;

BioLegend). Some naïve T cells were labeled with CellTrace™ CFSE reagent prior to stimulation. The CFSE dilution in culture T cells was used to indicate their proliferation. Cultured T cells collected at different hours were also analyzed by protein synthesis assay and western blotting as follows.

Protein synthesis assay

Cultured CD4 and CD8 T cells were collected at different time points (24 h, 48 h, 72 h, and 96 h) after stimulation with anti-CD3 and anti-CD28. One million cells per sample were used to detect nascent proteins according to the manufacture protocol (Protein Synthesis Assay kit, NO.601100, Cayman Chemical). Stained CD4 and CD8 T cells were accessed on a LSR II flow cytometer (Beckton Dickinson), and the data was analyzed using the FlowJo v10 software (Tree Star, Inc.).

Western blotting

CD4 and CD8 T cells were collected at different time points (0 h, 24 h, 48 h, and 72 h) after stimulation with anti-CD3 and anti-CD28. The whole cell extracts were isolated using lysis buffer (Bio-Rad). Proteins were separated by SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were treated with the blocking buffer for 2 h, and then incubated with the Foxo1 rabbit mAb (#2880, Cell Signal Technology) or β -actin mouse mAb (#12262, Cell Signal Technology). Horseradish peroxidase-linked antibodies to rabbit (#7074, Cell Signal Technology) or mouse (#7076, Cell Signal Technology) immunoglobulin G were used as secondary antibodies. Protein expression was detected by chemiluminescence.

Statistical analysis

Data were represented as mean $\pm$ SD and analyzed with Prism version 7.0a (GraphPad Software). The *p* values of graft survival were determined by the Mann-Whitney test. Other measurements were performed using unpaired Student's *t*-test. Differences were considered significant when *p* < 0.05.

RESULTS

Deletion of mTOR in T Cells induces long-term heart allograft survival

To determine the role of mTOR in regulating T cell responses in transplantation, BALB/c hearts were transplanted into *Mtor^{fl/fl}Cd4-Cre* (B6 background) and wild type (WT) B6 mice. As shown in Figure 1A, none of the *Mtor^{fl/fl}Cd4-Cre* mice rejected their BALB/c heart allografts (median survival time (MST) of >100 days; *n* = 5), whereas WT B6 mice rejected BALB/c hearts acutely (MST = 8.6 ± 0.55 days; *n* = 5). Histology of heart allografts harvested from *Mtor^{fl/fl}Cd4-Cre* recipients at day 8 post-transplant showed intact myocytes with less cellular infiltration when compared to those of heart allografts from WT recipients (Figure 1B). The number of splenocytes in *Mtor^{fl/fl}Cd4-Cre* recipients at day 8 post-transplant was significantly less expanded than that in WT recipients (Figure 1C). Hence, mTOR deletion in T cells abrogates their ability to reject heart allografts.

mTOR is required for Tfh cell response in heart transplantation

To define why mTOR deletion in T cells prevents heart allograft rejection, splenocytes were obtained from *Mtor^{fl/fl}Cd4-Cre* and WT B6 recipients at day 8 post-transplantation, followed by flow cytometry analysis. As shown in Figures 2A and 2B, CD62L⁻CD44⁺ effector T cells were dramatically expanded in WT recipients in response to heart transplantation. The frequencies of CD62L⁻CD44⁺ effector T cells within CD4⁺ and CD8⁺ cell populations in WT heart-transplant recipients were significantly higher than those in *Mtor^{fl/fl}Cd4-Cre* heart-transplant recipients. Moreover, transplantation of allogeneic hearts in WT but not in *Mtor^{fl/fl}Cd4-Cre* mice induced the generation of PD-1⁺CXCR5⁺ and BCL-6⁺CXCR5⁺ Tfh cells (Figures 2C and 2D) as well as the GL-7⁺B220⁺ germinal center (GC) B cells (Figures 2E and 2F). At day 14 post-transplantation, PNA⁺ GCs were formed in the spleens of WT, but not *Mtor^{fl/fl}Cd4-Cre*, heart-transplant recipients (Figure 2G). Therefore, mTOR is not only essential for the expansion of effector T cells, but also required for the generation of Tfh and GC B cells as well as the formation of GCs in transplantation.

mTOR promotes T cell proliferation and protein synthesis

To further define how mTOR affects T cell biology, we isolated CD4⁺ and CD8⁺ mTOR knock out (KO) T cells from naïve *Mtor^{fl/fl}Cd4-Cre* mice, and compared them with WT T cells regarding cell proliferation and protein synthesis upon in vitro activation by anti-CD3 and anti-CD28. To determine cell proliferation, T cells were labeled with CFSE prior to activation. As shown in Figure 3A, CD4⁺ and CD8⁺ mTOR KO T cells proliferated less than those of WT T cells upon activation. Protein synthesis was assessed by using O-Propargyl-puromycin (OPP) as a probe for labeling translating polypeptide chains, and using 5 FAM-Azide for detecting OPP-labeled proteins. We found that CD4⁺ and CD8⁺ mTOR KO T cells exhibited decreased protein synthesis after activation when compared with those of WT T cells (Figure 3B). Figure 3C shows that Foxo1 but not β -Actin expression was reduced in mTOR KO T cells compared to those of WT T cells, suggesting that mTOR selectively regulates protein production. Together, mTOR deletion in T cells inhibits their proliferation and protein synthesis.

mTOR in T cells promotes memory response to transplants

We next determined the effects of T cell-specific mTOR deletion on regulating memory response in transplantation. *Mtor^{fl/fl}Cd4-Cre* and WT B6 mice were transplanted with BALB/c ear skins. At more than 30 days post-skin graft rejection, the recipient mice were transplanted again with BALB/c heart allografts. At day 5 post-heart transplantation, spleens and heart allografts were harvested for analysis (Figure 4A). The numbers of splenocytes in WT and *Mtor^{fl/fl}Cd4-Cre* transplant recipients were both expanded compared to those of naïve WT and *Mtor^{fl/fl}Cd4-Cre* mice (Figure 4B). The frequency of CD62L⁻CD44⁺ effector T cells within CD4⁺ splenocytes in WT transplant recipients was significantly higher than that in *Mtor^{fl/fl}Cd4-Cre* transplant recipients (Figures 4C and 4D). Histology of heart allografts from *Mtor^{fl/fl}Cd4-Cre* recipients showed intact myocytes with less cellular infiltration than those in heart allografts from WT recipients. Immunofluorescence staining of CD3 showed less T cell infiltration in heart allografts from *Mtor^{fl/fl}Cd4-Cre* recipients

than that of WT recipients (Figure 4E). Thus, mTOR deletion in T cells impairs memory response to transplants.

Ablation of both mTOR and Stat3 in T cells abrogates memory response to transplants

To define the key regulators driving the memory response to transplants, we first transplanted BALB/c ear skins onto *Mtor^{fl/fl}Cd4-Cre*, *Stat3^{fl/fl}Cd4-Cre*, *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre*, and WT mice, all of which have a B6 background. As shown in Figure 5A, among different groups, *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* mice exhibited the least potency in rejecting skin allografts (MST = 17.17 ± 0.75 days; n = 6). Skin graft survival was also prolonged on *Mtor^{fl/fl}Cd4-Cre* (MST = 14.50 ± 1.65 days; n = 10) and *Stat3^{fl/fl}Cd4-Cre* (MST = 15.50 ± 0.55 days; n = 6) mice when compared those on WT mice (MST = 9.33 ± 0.52 days; n = 6).

Recipient mice were transplanted again with BALB/c hearts more than 30 days after BALB/c skin graft rejection. Donor skin-sensitized WT recipients quickly rejected the heart allografts (MST = 5.40 ± 0.55 days; n = 5), whereas heart allograft survival was prolonged on donor skin-sensitized *Mtor^{fl/fl}Cd4-Cre* (MST = 19.50 ± 10.93 days; n = 6) and *Stat3^{fl/fl}Cd4-Cre* (MST = 17.80 ± 1.30 days; n = 5) mice. Most strikingly, none of the donor skin-sensitized *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* mice rejected the heart allografts (MST >100 days; n = 6) (Figure 5B). We then analyzed the splenocytes at day 20 post-heart transplantation. The number of splenocytes and the frequency of CD4⁺ effector cells in *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* recipients was significantly less than those of *Stat3^{fl/fl}Cd4-Cre* recipients (Figures 5C and 5D). The frequency of CD8⁺ effector cells in *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* recipients was significantly less than those of *Mtor^{fl/fl}Cd4-Cre* and *Stat3^{fl/fl}Cd4-Cre* recipients (Figure 5D). Histology of heart allografts from skin-sensitized *Mtor^{fl/fl}Stat3^{fl/fl}Cd4-Cre* recipients showed intact myocytes with minimal cellular infiltration at day 120 post-heart transplantation (Figure 5E). Taken together, deletion of both mTOR and Stat3 in T cells abrogates memory response to transplants.

DISCUSSION

The molecular mechanisms underlying allogeneic T cell responses have not been adequately explored. In the present work, we found that all heart allografts survived more than 100 days in mice when mTOR was deleted in T cells. Further mechanistic investigation revealed that mTOR in T cells promoted the expansion of CD4⁺ and CD8⁺ effector cells, and was required for the generation of Tfh and GC B cells in response to heart transplantation. Moreover, in skin-sensitized recipients, although mTOR deletion alone in T cells only briefly prolonged the heart allograft survival, deletion of both mTOR and Stat3 in T cells induced long-term heart allograft survival. Therefore, mTOR is a key regulator that dictates primary and memory T cell responses in transplantation.

Previous reports showed that mTOR promotes the differentiation of effector T cells. In a transplantation setting, it is difficult to track the alloantigen-specific effector T cells unless a TCR transgenic system is used (16, 17). Nevertheless, we detected a dramatic increase of CD62L⁻CD44⁺ effector T cells in WT heart-transplant recipients. Although the antigen-specificity of those cells remains unknown, they increased in response to heart

transplantation. Strikingly, deletion of mTOR in T cells significantly inhibited such effector T cell expansion. In WT recipients, heart transplantation induces Tfh cell development. In the absence of mTOR in T cells, Tfh cells were barely detectable in both naïve and heart-transplant recipient mice regardless of using PD1⁺CXCR5⁺ or BCL6⁺CXCR5⁺ as markers. Consequently, mTOR deletion in T cells also inhibited the GC B cell generation and GC formation in response to heart transplantation.

mTOR forms two complexes with distinct components and functions: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Essential downstream molecules of mTORC1 include S6K, eLF4E, HIF1 α , and other proteins. Downstream molecules of mTORC2 are Akt, PKC, and SGK (19). mTORC1 but not mTORC2 is sensitive to an mTOR inhibitor rapamycin. Using infection/vaccination models, Araki et al. have shown that rapamycin promotes memory CD8⁺ T-cell generation through inhibiting mTORC1 (15). Published adjacent to Araki et al.'s paper, Pearce et al. have used rapamycin as an inducer of fatty acid oxidation to increase memory CD8⁺ T-cell generation (20). Later, Rao et al. have shown that rapamycin also enhance Eomes expression to promote memory CD8⁺ T-cell generation (21). These previous reports demonstrate that inhibition of mTOR by rapamycin promotes memory response (Figure 6A). Of note, as mentioned in the online methods in Araki et al.'s paper, a low dose of rapamycin (75 μ g/kg) is used during the T-cell expansion phase to promote memory CD8⁺ T-cell generation, because higher dose of rapamycin (600 μ g/kg) inhibits T-cell response (15).

We found that mTOR deletion in T cells reduces memory response to transplants, which is different from rapamycin-mediated mTOR inhibition that enhances memory CD8⁺ T-cell response to infections. This difference may be explained by the following reasons. First, pharmaceutical inhibitors have dose-dependent effects. As mentioned above, high-dose rapamycin inhibits T-cell response rather than promotes memory CD8⁺ T-cell generation. Second, mTOR activity within mTORC2 is insensitive to acute rapamycin treatment. Third, rapamycin has various effects on the immune system (22), which may indirectly influence T-cell response. Fourth, it is unknown whether low-dose rapamycin also enhances memory CD4⁺ T-cell response. Of note, CD4⁺ but not CD8⁺ T cells are central to the immunological rejection of transplanted organs (17). In our genetic deletion model, we used the CD4-CRE and mTOR-flox system to conditionally knockout mTOR (eliminates both mTORC1 and mTORC2) in CD4⁺ and CD8⁺ T cells, but not in other cells. Taken together, the regulation of memory response by low-dose rapamycin is incomparable to that of mTOR deletion in T cells.

mTOR deletion and rapamycin have some similar and different effects on T-cell responses. First, we and others found that mTOR KO T cells proliferated much less than WT T cells upon activation (14). Rapamycin also suppresses T-cell proliferation and is an effective immunosuppressant (23). Second, we found that mTOR deletion in T cells abrogated the generation of Tfh cells in response to heart transplantation. Rapamycin treatment has also been shown to impair the formation of Tfh cells during acute viral infection (24). Third, mTOR KO CD4⁺ T cells differentiate into inducible regulatory T cells, but fail to become Th1, Th2, or Th17 cells when stimulated under appropriate skewing conditions (14). Rapamycin also can promote the generation of regulatory T cells (25–27), and inhibit Th1

and Th17 cell differentiation (26, 28). Nevertheless, rapamycin has been shown to promote Th2 cell differentiation (29). It remains unknown why mTOR deletion and rapamycin-mediated mTOR inhibition exert different effects on Th2 cell differentiation. Fourth, mTOR is critical for cell metabolism (19). Increasing fatty acid oxidation is one of the mechanisms by which rapamycin increases memory CD8⁺ T-cell generation (Figure 6A) (20). In contrast to rapamycin, mTOR deletion in T cells reduces memory response to transplants. Therefore, mTOR deletion may be different from rapamycin-mediated mTOR inhibition in regulating fatty acid oxidation in T cells. It would be interesting to further investigate how mTOR deletion affects T-cell metabolism. Moreover, mTOR deletion in T cells exhibit potent effects on abrogating heart allograft rejection and reducing allogeneic memory response. This raises a question that whether better mTOR inhibitors need to be developed to replace rapamycin. Indeed, inhibitors that target both mTORC1 and mTORC2 have been developed (30), and their therapeutic effects on preventing transplant rejection need to be investigated.

We briefly investigated how mTOR affects T cell biology in vitro. Deletion of mTOR in T cells dramatically inhibits their proliferation and protein synthesis upon cell activation. By western blotting of Foxo1 and β -Actin, we found that Foxo1 but not β -Actin expression was reduced in mTOR KO T cells. Thus, mTOR selectively regulates protein production. Foxo1 is an essential regulator of CD8⁺ memory T cells (31, 32). Reduction of Foxo1 expression may be one of the mechanisms by which mTOR deletion impairs T cell memory.

Stat3 is a transcription factor, and regulates the expression of various genes in response to growth factors and cytokines. In the context of memory T-cell generation, Cui et al. have shown that the IL-21/IL-10–Stat3 pathway sustains the expression of BCL-6, SOCS3 and Eomes, and in turn promotes memory CD8⁺ T cell development after acute LCMV infection (Figure 6B). Stat3-deficient CD8⁺ T cells exhibit impaired ability to develop into central memory T cells (33). In consistent with this finding, we found that Stat3 deletion in T cells prolonged the heart allograft survival in donor skin-sensitized recipients, indicating that Stat3 deletion in T cells reduces memory response to transplants.

mTOR deletion in T cells reduces Stat3 phosphorylation during Th17 cell differentiation (14). However, in the context of memory T-cell response, it is unknown whether mTOR and Stat3 interact and influence each other. Ablation of both mTOR and Stat3 in T cells induced long-term heart allograft survival in donor skin-sensitized recipients. Thus, ablation of both mTOR and Stat3 in T cells is more potent than mTOR or Stat3 deletion alone to eliminate allogeneic memory response (Figure 6C), suggesting the existence of non-overlapping effects of mTOR and STAT3 on memory T-cell response.

In summary, mTOR in T cells promoted the primary and memory immune response to transplants. In particular, Tfh cell response was nearly completely absent in transplant recipients with T cell-specific mTOR deletion. Long-term heart allograft survival was also achieved in donor-skin sensitized recipients with T cell-specific deletion of both mTOR and Stat3. These findings provide fundamental understanding of allogeneic T cell responses at the molecular level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. mTOR deletion in T cells induces long-term heart allograft survival.

*Mtor^{fl/fl}*Cd4-Cre or WT B6 mice were transplanted with BALB/c hearts. A, The percentage of heart allograft survival after transplantation. $**p < 0.001$; Mann-Whitney test. B, H&E stained sections of heart allografts harvested from WT B6 and *Mtor^{fl/fl}*Cd4-Cre recipients at day 8 post-transplant. Three mice per group were analyzed, with ten graft sections per mouse. Two representative images are shown. C, number of splenocytes in WT B6 and *Mtor^{fl/fl}*Cd4-Cre recipients at day 8 post-transplant. Data are presented as mean $\pm$ SD (n = 5). $*p < 0.05$; unpaired student's t-test.

Figure 2. mTOR promotes effector and Tfh cell response to heart transplants.

Mtor^{fl/fl} Cd4-Cre or WT B6 mice were transplanted with BALB/c hearts (HTx groups) or left un-transplanted (Naïve groups). A and B, Frequencies of CD62L⁺CD44⁺ effector T (Teff) cells within CD4⁺ or CD8⁺ splenocytes in indicated groups at day 8 post-transplant. C and D, Frequencies of PD-1⁺CXCR5⁺ or BCL-6⁺CXCR5⁺ Tfh cells within CD4⁺ splenocytes in indicated groups at day 14 post-transplant. E and F, Frequencies of GL-7⁺B220⁺ GC B cells within splenocytes in indicated groups at day 14 post-transplant. G, Spleen sections from WT B6 and *Mtor^{fl/fl}* Cd4-Cre recipients at day 14 post-transplant were analyzed by immunofluorescence staining. Three mice per group were analyzed, with ten spleen sections per mouse. Two representative images show the PNA, IgM and CD4 expression. A-F, Data are presented as mean $\pm$ SD (n = 5). * p < 0.05; unpaired student's t-test.

Figure 3. mTOR promotes T cell proliferation and protein synthesis upon activation.

Naïve WT and mTOR knockout (KO) T cells (CD4⁺ or CD8⁺) were sorted from the splenocytes of naïve WT B6 and *Mtor*^{fl/fl} *Cd4-Cre* mice, followed by stimulation in vitro with anti-CD3 and anti-CD28. A, The overlay histograms display dilution of CFSE, indicating cell proliferation at indicated hours post-stimulation. B, O-Propargyl-puromycin (OPP) was used for labeling translating polypeptide chains and 5 FAM-Azide was used for detecting OPP-labeled proteins. The overlay histograms show protein synthesis in cells at indicated hours post-stimulation. C, Western plotting of Foxo1 and β-Actin in cells at indicated hours post-stimulation.

Figure 4. mTOR deletion inhibits T cell response to heart transplants in donor skin-sensitized recipients.

Mtor^{fl/fl} Cd4-Cre or WT B6 mice were transplanted with BALB/c ear skins. More than thirty days after skin graft rejection, recipients were transplanted again with BALB/c hearts. Splenocytes and heart allografts were harvested at day 5 post-heart transplantation for analysis. Un-transplanted naïve mice were used as controls. A, Schematic of the experimental design. B, Number of splenocytes in indicated groups. $n = 5$ per group. C and D, Frequencies of CD62L⁻CD44⁺ effector T (Teff) cells within CD4⁺ or CD8⁺ splenocytes in indicated groups. Data are presented as mean $\pm$ SD ($n = 5$). * $p < 0.05$; unpaired student's t -test. E, Heart allograft sections were analyzed by H&E staining or anti-CD3 mAb plus DAPI immunofluorescence staining. Three mice per group were analyzed, with ten graft sections per staining per mouse. Representative images are shown.

Figure 5. Ablation of both mTOR and Stat3 induces long-term heart allograft survival in donor skin-sensitized recipients.

WT B6, $Mtor^{fl/fl} Cd4-Cre$, $Stat3^{fl/fl} Cd4-Cre$, or $Mtor^{fl/fl} Stat3^{fl/fl} Cd4-Cre$ mice were transplanted with BALB/c ear skins. More than thirty days after skin graft rejection, recipients were transplanted again with BALB/c hearts. A, The percentage of skin allograft survival after skin transplantation. B, The percentage of heart allograft survival after heart transplantation in donor skin-sensitized mice. WT vs. $Mtor^{fl/fl} Stat3^{fl/fl} Cd4-Cre$ * $p < 0.01$; Mann-Whitney test. C, Number of splenocytes in indicated groups at day 20 post-heart transplant. Data are presented as mean $\pm$ SD (n = 5). * $p < 0.05$; unpaired student's t-test. D, Frequencies of CD62L⁻CD44⁺ effector T (Teff) cells within CD4⁺ or CD8⁺ splenocytes in indicated groups. Data are presented as mean $\pm$ SD (n = 5). * $p < 0.05$; unpaired student's t-test. E, The representative image shows an H&E stained section of heart allograft harvested from donor skin-sensitized $Mtor^{fl/fl} Stat3^{fl/fl} Cd4-Cre$ recipients at day 120 post-heart transplant.

Figure 6. Graphic summary of the regulation of memory T cell response by mTOR and Stat3.

A, mTOR forms two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Rapamycin promotes memory CD8⁺ T-cell generation through inhibiting mTORC1, increasing fatty acid oxidation (FAO), and enhancing Eomes expression. B, IL-21/IL-10–Stat3 pathway promotes memory CD8⁺ T-cell development through sustaining the expression of BCL-6, SOCS3, and Eomes. C, Ablation of both mTOR (eliminates mTORC1 and mTORC2) and Stat3 abrogates memory T (Tmem) response in transplantation.