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TITLE: High-Dose Post-Transplantation Cyclophosphamide to Induce Delayed Immune Tolerance After Reconstructive Transplantation

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14. ABSTRACT

The overall objective of this project is to understand mechanisms of delayed transplant tolerance as they specifically relate to VCA, and establish a donor bone marrow and PT/Cy-based protocol for the induction of delayed tolerance with minimal or only transient immunosuppression after reconstructive transplantation. Our central hypothesis is that a vascularized intragraft BM stromal micro-environment combined with PT/Cy treatment will promote immunoregulatory mechanisms that allow for establishing delayed tolerance and ultimately immunosuppression-free graft survival. We aimed to first determine the optimal time point for the application of PT/Cy after VCA and secondly evaluate whether further transplantation of additional exogenous donor bone marrow can augment the outcome of allograft survival in the context of donor chimerism (SPECIFIC AIM 1). The investigators were able to establish a reliable treatment protocol using rapamycin (5 mg/kg) combined with delayed PT/Cy in a mouse orthotopic hind limb transplantation model. The application of this treatment protocol leads to prolonged VCA survival (77.6 ±28.75 days) and donor-specific mixed chimerism (Avg: 2.04%; Range: 0.1%-6.49%). Further *in-vitro* studies are geared towards the role of memory T cells in rejection of VCA after delayed PT/Cy and the use of additional donor bone marrow transplantation combined with delayed PT/Cy is ongoing.

15. SUBJECT TERMS:
vascularized composite allotransplantation, delayed tolerance, post-transplantation cyclophosphamide, bone marrow transplantation.

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High-Dose Post-Transplantation Cyclophosphamide to Induce Delayed Immune Tolerance After Reconstructive Transplantation
PI: Gerald Brandacher, M.D. & Leo Luznik, M.D.

1. INTRODUCTION

Close to 40% of combat injuries sustained in OEF and OIF involved severe extremity and craniofacial trauma. Currently, despite the best reconstructive efforts by using native tissue these injuries are not only mutilating, but also frequently result in permanent disfigurement and morbidity. For most devastating injuries for which conventional reconstruction is not possible, vascularized composite allotransplantation (VCA) has become a viable alternative to reconstruct complex defects.

However, the life-long use of immunosuppressants and their associated medical toxicities remain one of the primary obstacles that curtail the wider use of VCA for reconstruction. These risks and side effects greatly compromise recipient quality of life and jeopardize the potential benefits of VCA. One promising strategy that addresses this challenge is induction of immune tolerance through combined donor bone marrow transplantation (dBMT) together with VCA. Donor antigen-specific immune tolerance without immunosuppression has been realized in small and large animal models. It was also recently realized in humans in the setting of living-related renal transplantation using simultaneous BM and organ transplantation to achieve mixed chimerism. However, the ability to perform combined dBMT and VCA from the same unrelated HLA-mismatched cadaveric donor due to the requirement of extensive preconditioning of the recipient is still limited. Therefore, there is currently no readily available clinical tolerance protocol for VCA. The novel concept of “delayed tolerance” offers compelling potential to bypass this limitation in VCA. In this scenario the recipient initially undergoes VCA transplantation with conventional immunosuppression, followed by conditioning and dBMT at a later stage post transplantation.

The overall goal of this proposal is to establish a dBMT and post-transplantation high-dose cyclophosphamide (PT/Cy)-based protocol for the induction of delayed tolerance with minimal or only transient immunosuppression for VCA and elucidate critical cellular and molecular mechanisms behind this novel strategy.

2. KEYWORDS

vascularized composite allotransplantation, delayed tolerance, post-transplantation cyclophosphamide, bone marrow transplantation.

3. ACCOMPLISHMENTS

During the first year of this project, a total of 52 successful VCA transplantations (i.e. mouse orthotopic hind limb transplantation) were performed across a full MHC mismatch from a Balb/c donor to a C57BL/6J recipient animal. Hind limb recipients were treated with conventional immunosuppression to prevent VCA rejection prior to receiving the delayed post-transplant cyclophosphamide-based treatment protocol (delayed PT/Cy, **Figure 1**).

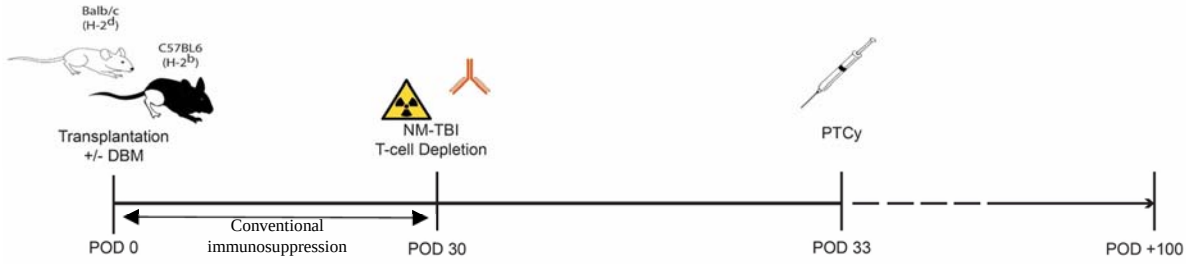

Figure 1: Treatment regimen.

A. Major Goals

The major goals of this project for Year 1 are:

Major Task 1: Identify the time delay between VCA and start of post-transplantation conditioning that leads to successful vascularized composite allograft acceptance and stable chimerism.

Major Task 2: Optimize the delayed PTCy-based tolerance regimen that results in improved acceptance of VCA.

Table 1: Progress against the SOW.

Task	**Start Date**	**End Date**	**% Complete**	**Comments**
Major Task 1	October 2017	October 2018	35%	Delayed – *In-vivo* and *in-vitro* experiments are ongoing using rapamycin (5 mg/kg) combined with delayed application of PTCy.
Major Task 2	May 2017	-	25%	Ongoing – Experiments using rapamycin combined with delayed PTCy and donor bone marrow augmentation are ongoing.

B. Accomplishment of Goals

Major Task 1 - Subtask 1: Obtain IACUC and ACURO approval for the mouse orthotopic hind limb transplantation and delayed induction treatment.

We successfully obtained IACUC and ACURO approval on (10/19/2017) and on (2/12/2018), respectively. Unfortunately, this represents a delay in the approval process which ultimately lead to a delayed start of the *in-vivo* experiments outlined by the SOW.

Major Task 1 - Subtask 2: Identify the time delay between VCA and start of post-transplantation conditioning that leads to successful vascularized composite tissue acceptance and stable mixed chimerism.

During Year 1 of the performance period we performed mouse orthotopic hind limb transplantation across a full MHC mismatch. As outlined in the SOW, animals were initially treated with cyclosporine A (25 mg/kg) for 30 days prior to the scheduled application of the delayed PT/Cy treatment protocol (**Table 2**, Group 3). As shown by **Figure 2A**, the use of cyclosporine A did not lead to rejection-free allograft survival in this murine model. These unexpected preliminary findings required the adaptation of the conventional immunosuppressive treatment protocol to achieve rejection-free graft survival for the first 30 days. In order to correct this issue a dose escalation study was performed using various doses of cyclosporine A and rapamycin - an alternative immunosuppressive agent. Ultimately, the use of rapamycin at a dose of 5mg/kg lead to rejection-free VCA survival for 30 days and thus demonstrated efficacy to be used as the conventional immunosuppressive agent in this specific murine model of VCA (**Figure 2B)**.

Table 2: Study group outline.

Subtask 2: Identify the time delay between VCA and start of post-transplantation conditioning that leads to successful vascularized composite tissue acceptance and stable mixed chimerism:
• Group 1: Condition on POD +1 after VCA and PTCy on POD +4
• Group 2: Condition on POD +10 after VCA and PTCy on POD +13
• Group 3: Condition on POD +30 after VCA and PTCy on POD +33

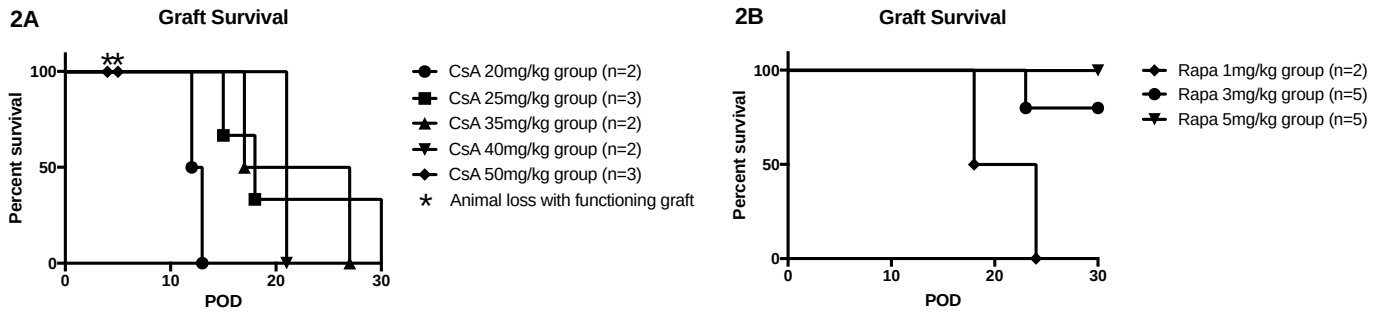

Figure: **2A** Graft survival of cyclosporine A treatment; **2B** Graft survival of rapamycin treatment.

After determining the optimal treatment protocol to achieve rejection-free VCA survival, hind limb allograft recipients were enrolled in Group 3 in which delayed PTCy treatment was successfully administered on POD 30. Daily clinical assessment and photo documentation was performed (**Figure 3A**). As shown in **Figure 3B**, the application of delayed PTCy after 30 days of rapamycin treatment resulted in a VCA graft survival of 77.6 ±28.75 days. Chimerism levels were ranging from ranging from 0.1% to 6.49% with an average of 2.04% (**Figure 3C**).

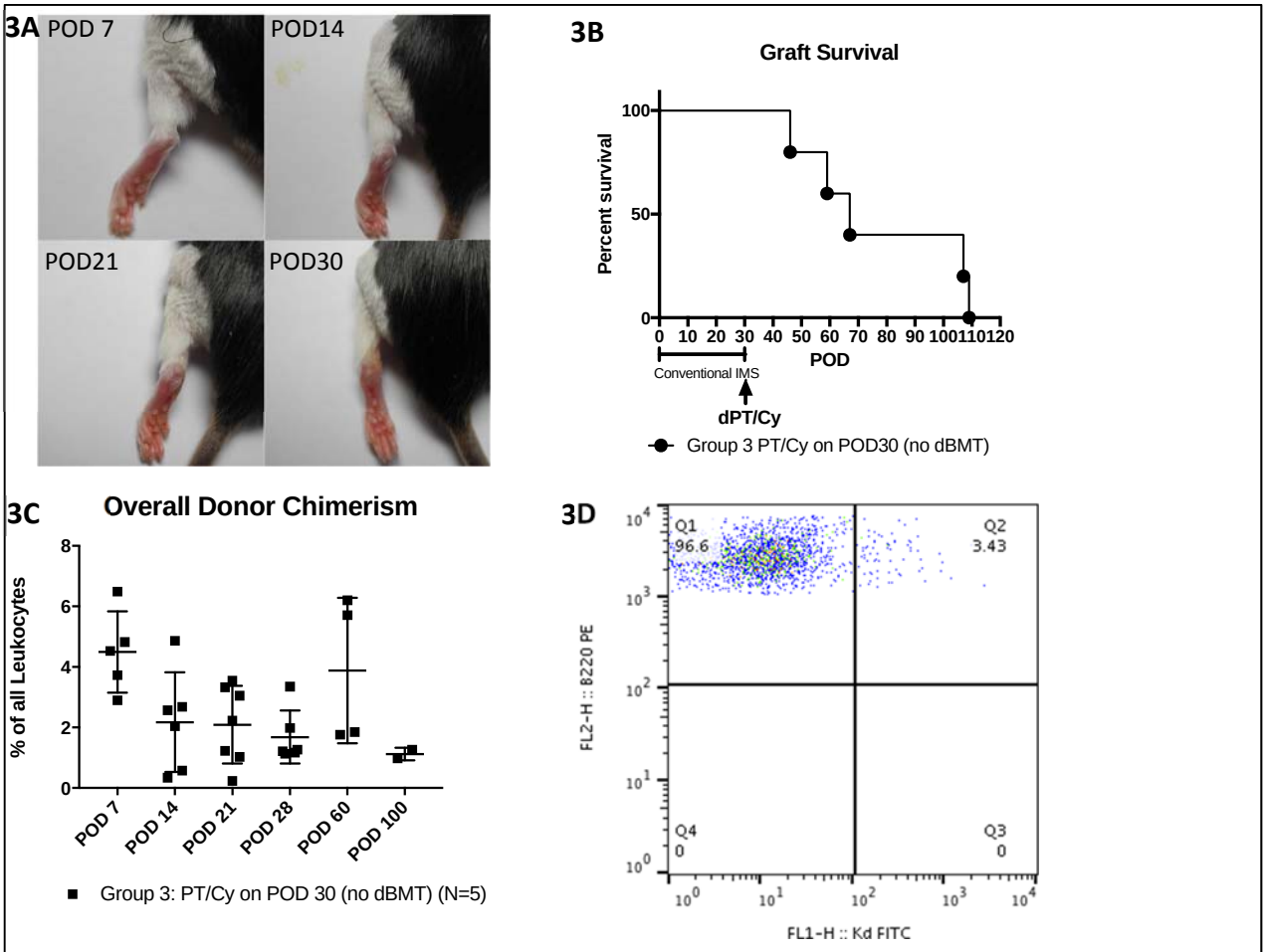

Figure 3: **3A:** Clinical images of VCA allograft treated with rapamycin (5 mg/kg); **3B:** Graft survival after rapamycin for 30 days combined with PT/Cy (no dBMT); **3C:** Overall donor-specific multi-lineage mixed chimerism in VCA recipient. **3D:** Exemplary flow cytometric analysis of donor-derived B cell chimerism.

Major Task 2 - Subtask 1: Evaluate the effects of delayed donor bone marrow administration on VCA acceptance and stable mixed chimerism.

Experiments in Major Task 2 - Subtask 1 are currently ongoing. A total of 5 mice were enrolled into Group 3, *receiving delayed PTCy treatment combined with donor bone marrow augmentation* after 30 days of rapamycin (5mg/kg). As shown by **Figure 4A**, the application of delayed PTCy after 30 days of rapamycin treatment plus bone marrow adminstration resulted in a graft survival of 74 ± 17.6 days.

Major Task 2 - Subtask 2: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of fresh donor bone marrow and PT/Cy.

In these ongoing experiments we have performed donor-specific multi-lineage mixed chimerism analysis in all animals enrolled in Group 3 receiving a VCA allograft plus 30 days of rapamycin, delayed PTCy and *bone marrow administration*. **Figure 4B** shows that donor-derived hematopoietic chimerism ranges from 0.4% to 7.11%.

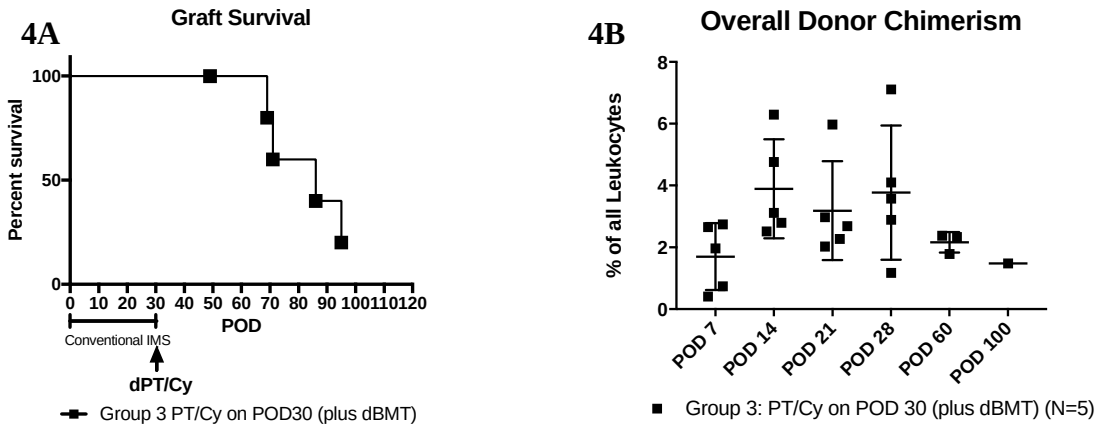

Figure 4: 4A Allograft survival; 4**B** Chimerism analysis.

In addition to mixed chimerism analysis, we performed flow cytometry-based analysis of T memory cell (T_{mem}) frequency in animals enrolled in Group 3 with and without donor bone marrow transplantation (dBMT). As shown in **Figure 5**, animals with robust allograft rejection demonstrate a higher level of T_{mem} frequency after the application of delayed PTCy.

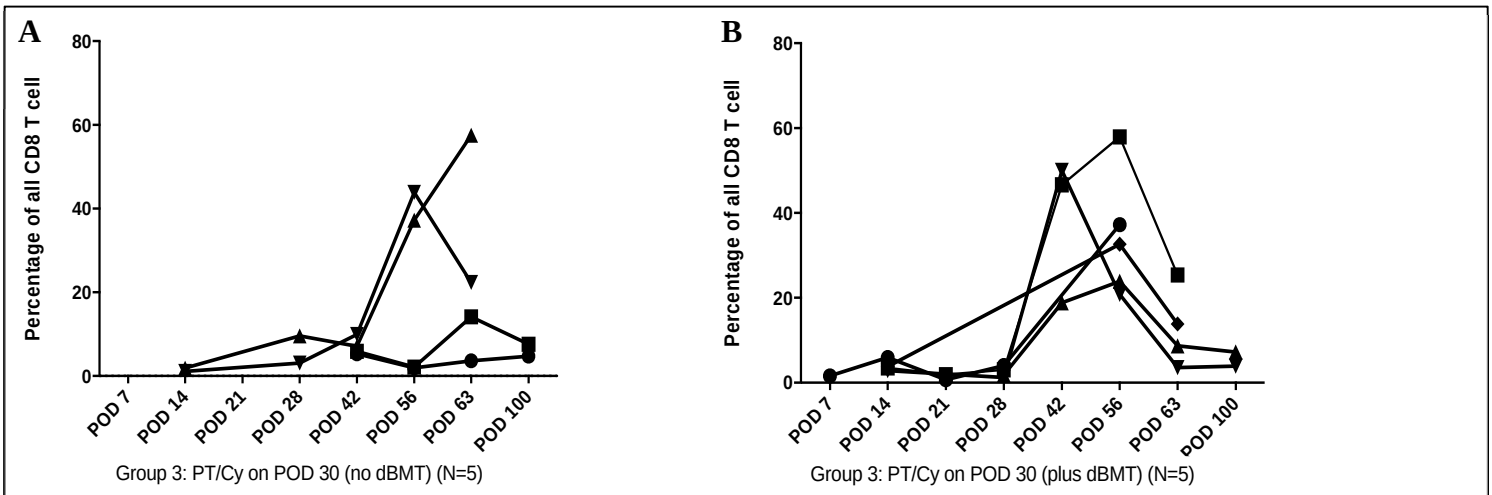

Figure 5: A CD8 T memory cells in Group 3 (no BM); **B** CD8 T memory cells in Group 3 (with BM)**.**

Significant results or key outcomes

The investigators successfully implemented a model system allowing to test the efficacy of delayed application of PTCy with and without donor bone marrow transplantation to induce long-term immunosuppression-free allograft survival in VCA recipients. This model system allows to investigate the impact of delayed PTCy on immunologic tolerance after conventional immunosuppression as well has given the investigators the opportunity to further study the mechanisms of donor bone marrow engraftment and immune tolerance in this murine model of VCA.

C. Training and Professional Development

Task 1 & 2 of the SOW have provided the PI's with the opportunity to solidify the training of the involved research fellows with regards to optimizing both microsurgical technique (mouse heterotopic hind limb transplantation model) as well as advanced *in-vitro* assays for the assessment of chimerism and memory T cell analysis.

D. Result Dissemination

Nothing to report

E. Future plan

During Year 2 of the performance period, experiments will be conducted according to the SOW. Specifically, we aim to increase the numbers of animals to be enrolled in Group 1 and 2 of both Major Task 1 and 2 and thereby assure timely completion of the proposed experiments.

4. IMPACT

A. Impact on the Development of the Principal Discipline(s) of the Project

The development of this animal models and the specific treatment protocol will allow the investigators and the community of transplant specialists to study the mechanisms involved in induction of delayed allograft tolerance after the use of conventional immunosuppression and hence will be indispensable to further advancement of the field of VCA. The results obtained by this study will allow for the development of specific, targeted, and clinically applicable treatment modalities for delayed induction of tolerance in VCA.

B. Impact on Other Disciplines

A better understanding of delayed tolerance induction along with the development of clinically applicable protocols, will not only contribute greatly to the advancement of the field of reconstructive transplantation but also be applicable to other types of solid organ transplantation.

C. Impact on Technology Transfer

Nothing to Report

D. Impact on Society beyond Science and Technology

Nothing to Report

5. CHANGES/PROBLEMS

The following two factors have led to a delay in performance of *in-vivo* experiments during Year 1 of the performance period:

1. Delayed approval of IACUC animal research protocol leading to delayed ACURO approval.
2. Ineffective immunosuppressive activity of cyclosporine A in the murine orthotopic hind limb transplantation model in C57BL6 mice.

A. Changes in Approach and Reasons for Change

We successfully established the use of rapamycin at 5 mg/kg as an effective alternative conventional immunosuppressant to achieve VCA survival for 30 days post transplantation. This change was necessary due to the ineffectiveness of cyclosporine A in achieving VCA graft survival.

B. Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them

Both factors stated above have led to a delay in performance of experiments. However, we have taken corrective action to make up for the time delay by devoting additional personnel to perform *in-vivo* experiments. We therefore expect to make up for lost time during Year 2 of the performance period and complete murine experiment during that time.

C. Changes that had a Significant Impact on Expenditures

Nothing to Report

D. Significant Changes in Use or Care of Human Subjects, Vertebrate Animals, Biohazards, and/or Select Agents

Nothing to Report

6. PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

No changes

B. Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period

Nothing to Report

C. Other organizations involved as partners

Nothing to Report