AWARD NUMBER: W81XWH-17-1-0280

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

PRINCIPAL INVESTIGATOR: Gerald Brandacher MD

CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

REPORT DATE: October 2020

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 020	Annual Technical Report	15Sep2019-14Sep2020

4. TITLE AND SUBTITLE
High-Dose Posttransplantation Cyclophosphamide to Induce Delayed Immune Tolerance

After Reconstructive Transplantation

5a. CONTRACT NUMBER
W81XWH-17-1-0280

5b. GRANT NUMBER
Log: RT160085/ 127076

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Gerald Brandacher M.D. & Leo Luznik, M.D

E-Mail: gbranda2@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
Department of Plastic and Reconstructive
Surgery, VCA lab, 720 Rutland Ave,
Baltimore 21205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Developing novel treatment concepts to minimize/avoid immunosuppression by induction of immune tolerance represents the prime task in the field of transplantation. Immunosuppression-free allograft survival has been achieved in several small and large animal models as well as in humans in living-related combined kidney and donor bone marrow transplantation via transient or stable mixed hematopoietic chimerism. This is a concept of particular interest in VCA, as component grafts may inherently contain vascularized donor bone marrow and thus a vital bone marrow niche home to donor-derived hematopoietic progenitor cells. However, as living-related transplantation is ethically precluded in VCA, reconstructive transplantation is limited to cadaveric donors and thus extensive pre-transplant preconditioning is not feasible. Recently, we were able to demonstrate immune tolerance in mice using a peri-transplant induction regimen based on high-dose post-transplantation cyclophosphamide treatment (PT/Cy). In the underlying novel approach, we aim to apply the PT/Cy treatment protocol after the use of conventional immunosuppression to induced a state of "delayed tolerance" in an attempt to bypass limitation of cadaveric donor settings in VCA.

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	17	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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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 bone marrow (BM) transplantation together with VCA. However, the use of living-related donors is ethically precluded in VCA. Hence, reconstructive transplantation is limited to deceased donors, which prevents the ability to perform extensive recipient preconditioning prior to transplantation due to a minimal time window between VCA procurement and transplantation. The novel concept of "delayed tolerance" offers compelling potential to bypass this limitation in VCA, but its mechanisms remain unclear.

Thus, the overall goal of this proposal is to establish a donor BM (dBM) 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 this year, a total of 40 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 animals. Hindlimb recipients received the enhanced treatment protocol including delayed PT/Cy with adjunctive treatment components (i.e: additional T cell depletion, additional donor bone marrow transplantation and fludarabine treatment). Furthermore, in Aim 2 advanced flow analysis was performed in long-term survivors. **Figure 1** shows all tested enhanced treatment concepts. **Figure 2** shows tested combinations of delayed PT/Cy with adjunctive treatment components and cryo-preserved bone marrow.

Figure 1: Alternative approaches - dPT/Cy with adjunctive therapy.

- **1A:** dPT/Cy on POD 10 with additional ATG on POD 20.
- **1B:** dPT/Cy on POD 10 with additional ATG on POD 20 and total 200mg/kg fludarabine on POD 7/9.
- **1C:** dPT/Cy on POD 30 with additional ATG on POD 40.
- **1D:** dPT/Cy on POD 30 with additional ATG on POD 40 and total 200 mg/kg fludarabine on POD 27/29.
- **1E:** dPT/Cy on POD 30 with additional ATG on POD 40 and total 400 mg/kg fludarabine on POD 27/29 plus infusion of double amount of donor bone marrow.

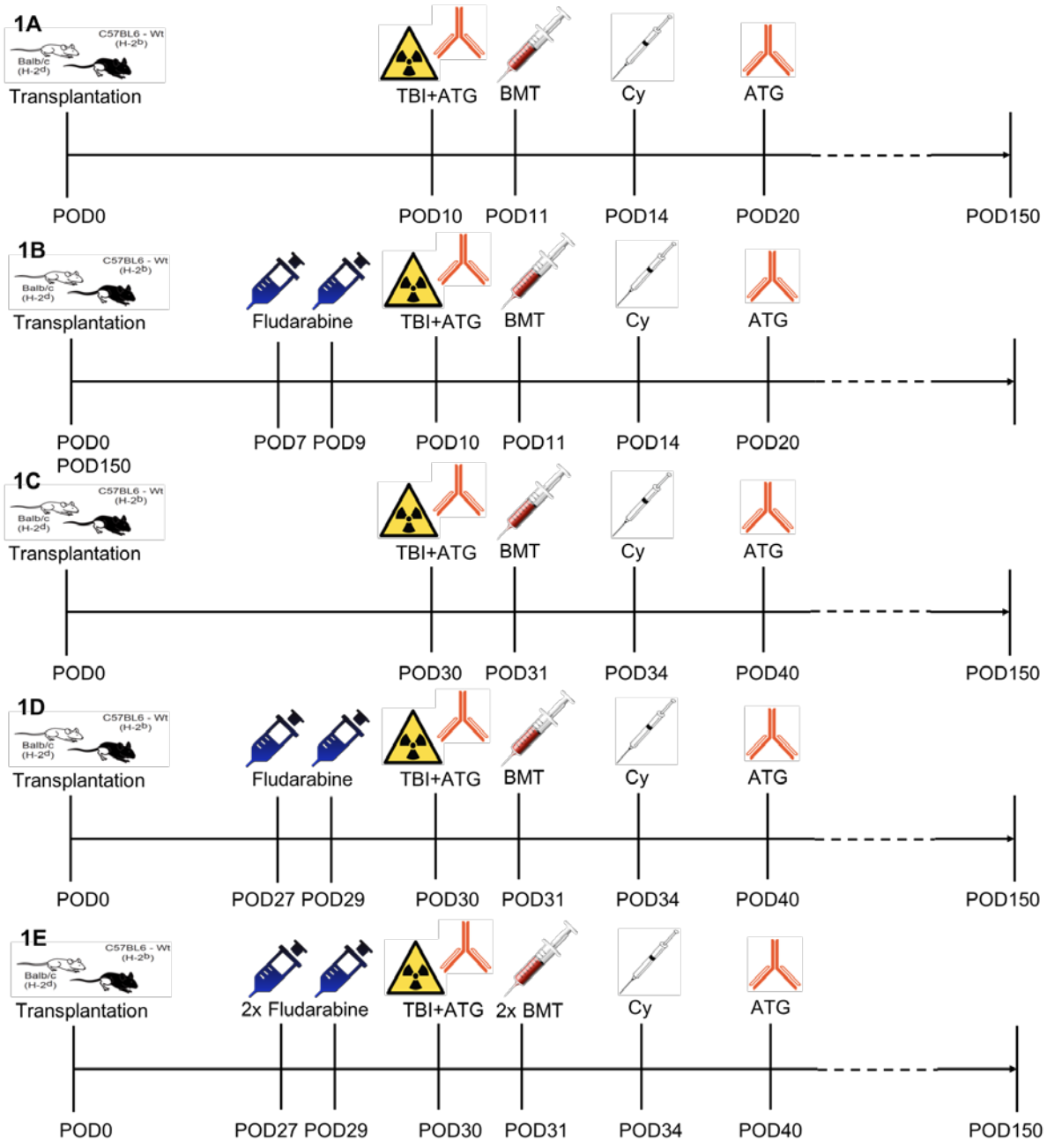
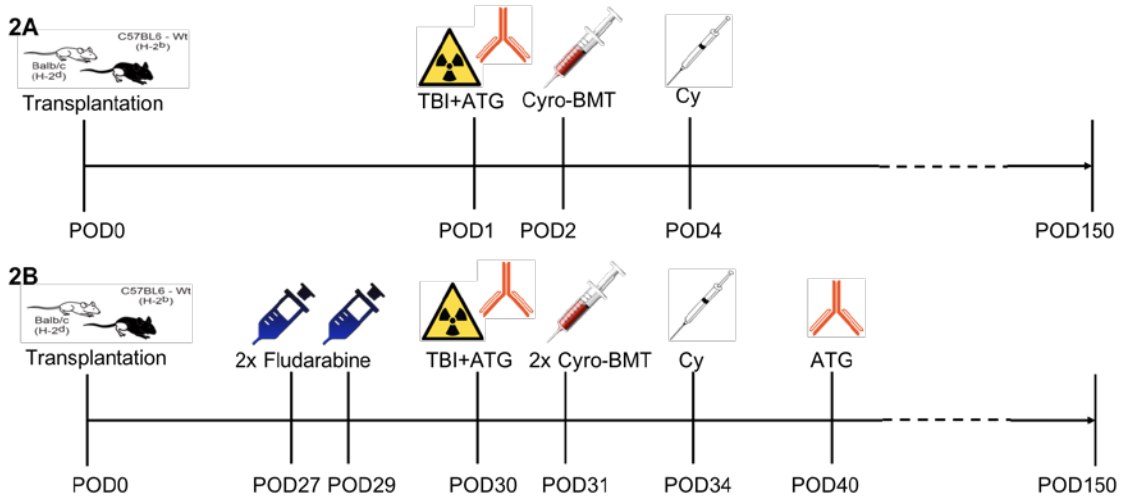


Figure 2: Alternative approaches - dPT/Cy with adjunctive therapy and cryopreserved dBM.

- **2A**: dPT/Cy on POD 1 using cryo-preserved donor bone marrow
- **2B**: dPT/Cy on POD 30 with additional ATG on POD 40 and total 400 mg/kg fludarabine on POD 27/29 plus infusion of cryo-preserved donor bone marrow



A. Major Goals

The major goals of this project for Year 3 are to accomplish work under the following Aims and Major Tasks:

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

Aim 1, Major Task 2:
Optimize the delayed PTCy-based tolerance regimen that results in improved acceptance of VCA.

Aim 2, Major Task 4:
Use Flow Cytometry, Computational Analysis, and high throughput sequencing to analyze the mechanisms of tolerance and the TCR repertoire in delayed tolerance induction via PT/Cy.

Aim 3, Major Task 5:
To establish optimal host conditioning for delayed tolerance induction and VCA in miniature swine model.

Table 1: Progress against the SOW

Specific Aim 1: To test and optimize efficacy of a delayed PTCy-based tolerance regimen in clinically relevant rodent VCA model	**Timeline** Months	**Completion (%)**
Major Task 1: Identify the time delay between VCA and start of post-transplantation conditioning that leads to successful vascularized composite tissue acceptance and stable chimerism?		
Subtask 1: Obtain IACUC and ACURO approval for the mouse orthotopic hind limb transplantation and delayed induction treatment.	1-3	100
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	3-9	100
Subtask 3: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of PT/Cy.	3-9	100
Major Task 2: Optimize delayed PTCy-based tolerance regimen that results in improved acceptance of VCA?	6-12	100
Subtask 1: Evaluate the effects of delayed donor bone marrow administration on VCA 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	6-12	100
Subtask 2: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of donor bone marrow and PT/Cy.	6-12	100
Major Task 3: Does fresh versus cryopreserved donor bone marrow affect the outcome of VCA allograft survival after delayed PTCy-based tolerance regimen?	9-12	95
Subtask 1: Compare the efficacy of cryopreserved donor bone marrow on VCA 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	9-12	95

Subtask 2: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of PT/Cy. Major Task 1-3 will require a total of 189 animals for completion with an additional 11 animals as bone marrow donors and as replacement of animal loss due to death or other study non-related reasons. Therefore, a grand total of 200 mice will be needed in Year 1.	9-12	95
Milestone #1: Establishing the most optimal timing of conditioning and donor bone marrow transplantation to promote immune tolerance, VCA survival and stable mixed chimerism.	3-12	
Specific Aim 2: To characterize mechanisms required to establish delayed tolerance for simultaneous composite tissue and delayed BM allotransplantation using high-dose PTCy.	12-24	25
Major Task 4: Use Flow Cytometry, Computational Analysis, and high throughput sequencing to analyze the mechanisms of tolerance and the TCR repertoire in delayed tolerance induction via PT/Cy.		
Subtask 1: Examine expression of exhaustion markers on T cells using flow cytometry. To accomplish the goals in Subtask 1 we will perform studies in interventional PT/Cy treated animals and compare them to untreated controls. • Interventional Group: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24) • Control Group, untreated: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24)	3-24	75
Subtask 2: Perform computational flow cytometry analysis To accomplish the goals in Subtask 2 we will perform studies in interventional PT/Cy treated animals and compare them to untreated controls. • Interventional Group: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24) • Control Group, untreated: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24)	12-24	75
Subtask 3: Conduct functional studies To accomplish the goals in Subtask 3 we will perform studies in interventional PT/Cy treated animals and compare them to untreated controls. • Interventional Group: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24) • Control Group, untreated: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24)	18-24	75
Subtask 4: Analyze the TCR repertoire of donor and recipient T cells To accomplish the goals in Subtask 4 we will perform studies in interventional PT/Cy treated animals and compare them to untreated controls. • Interventional Group: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24) • Control Group, untreated: N=12 recipients (strain: C57BL6) and donors (strain Balb/c), respectively; Total N=24) Major Task 4 will require a total of 192 animals for completion with an additional 8 animals as replacement of animal loss due to death or other study non-related reasons. Therefore, a grand total of 200 mice will be needed in Year 2.	18-24	30
Milestone #2: Manuscript on delayed PTCy-based tolerance regimen in a translational rodent VCA model and associated mechanisms	18-24	
Specific Aim 3: To translate and optimize efficacy of a delayed PT/Cy-based tolerance regimen in a clinically relevant large animal model (MGH miniature swine) for VCA.	24-36	5%
Major Task 5: To establish optimal host conditioning for delayed tolerance induction and VCA in miniature swine model.	24-36	5%
Subtask 1: Obtain IACUC and ACURO approval for swine heterotopic hind limb transplantation and delayed induction treatment.	24-26	50%
Subtask 2: Perform heterotopic hind limb transplantation in swine using a delayed tolerance protocol as determined by the results of AIM 1 and 2.	26-36	

Subtask 3: Examine the role of the degree and durability of chimerism on allograft survival in VCA in the swine model.	26-36	
Subtask 4: Define systemic and intragraft cellular and molecular signatures and profile changes that will serve as "biomarkers" of robust delayed tolerance induction for future clinical translation. To accomplish Major Task 5, we will perform swine heterotopic hind limb transplantation using the most favorable and least toxic protocol as determined by studies in Aim 1 and 2. In case that less then 3/3 animals engraft we will employ a more intense Regimen 2 and Regimen 3 if engraftment fails when using Regimen 2. In case of successful engraftment in any of these cohorts an additional 3 animals will be performed in this group. Tissue samples will be obtained simultaneously and sequentially in all groups and thus all subtasks in Major Task 5 may be accomplished. The MGH Miniature Swine strain will be used for both donor and recipient animals. The total number will be a minimum of 6 and a maximum of 12 recipients and a minimum of 3 and a maximum of 6 donor animals with a total of 18 animals to accomplish experimental goals in Major Task 5.	26-36	
Milestone #3: Manuscript on the establishment of protocol in miniature swine model of VCA.	30-36	

B. Accomplishment of Goals

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

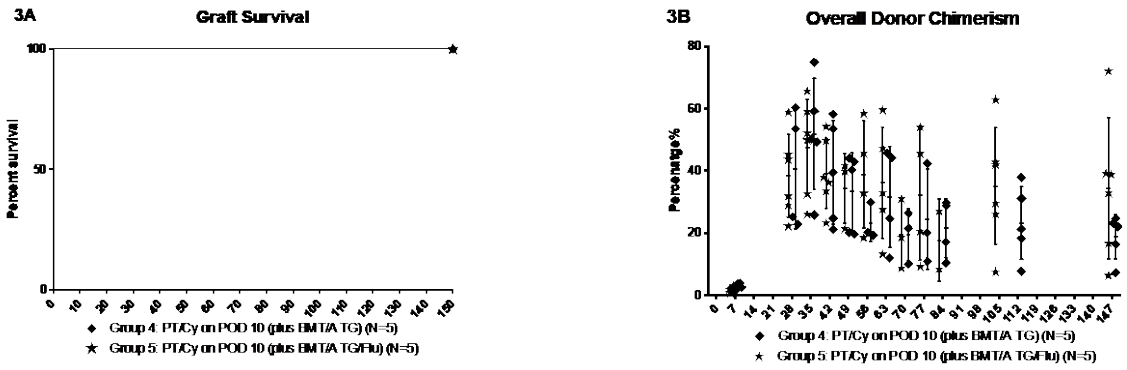
The originally proposed treatment had failed to accomplish the primary goal of successful vascularized composite allograft acceptance and stable chimerism under this Major Task. Therefore, alternative approaches as proposed in the grant narrative had to be developed and tested. These enhanced treatment concepts include the combination of dPT/Cy with adjunctive treatment components (i.e.: additional T cell depletion, additional dBMT and fludarabine treatment at various time points) as outlined by **Figure 1** and **Table 2 & 3**.

Table 2: Delayed PT/Cy on POD 10 – Enhanced Treatment Concepts.

Group Number	Treatment	N
Group 4	Rapamycin + dPT/Cy +dBMT+ Anti-Thy1.2 (POD 20)	5
Group 5	Rapamycin + fludarabine+dPT/Cy +dBMT+ Anti-Thy1.2 (POD 20)	5

Initial results were obtained in animals receiving the combination of dPT/Cy and dBMT with T cell depletion alone or additional fludarabine and T cell depletion on POD 10. This significantly improved both allograft survival and chimerism levels in both groups. Donor-specific mixed chimerism analysis for Group 4 and 5 shows an average of 23.40% (range: 0.52%-59.03%) of donor-specific chimerism in Group 4 and 27.11% (range: 1.77%-74.92%) in Group 5 (**Figure 3**).

Figure 3: Delayed PT/Cy on POD 10 – Alternative Approaches. Allograft survival (**3A**) and donor-specific mixed chimerism (**3B**) in Groups 4 and 5.



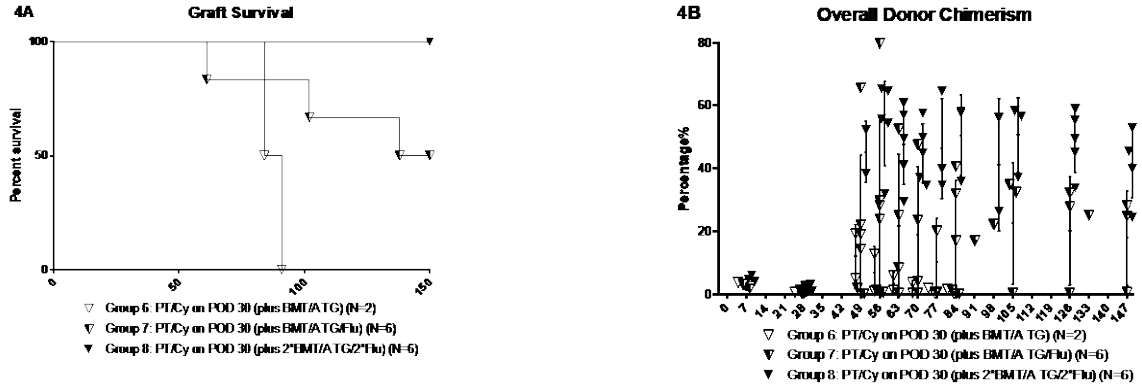
Data obtained from experiments in Group 4 & 5 encouraged the application of adjunct treatment 30 days after transplantation (Groups 6-9/POD 30). As shown in **Table 3** and illustrated by **Figure 1** repeated T cell depletion using anti-Thy-1.2 alone on POD 40 (Group 6, **Figure 1C**); fludarabine (POD 27 & 29) and repeated T cell depletion using anti-Thy-1.2 on POD 40 (Group 7, **Figure 1D**); and additional doses of fludarabine (POD 25, 26, 27 & 28) combined with an additional dose of donor bone marrow and repeated T cell depletion using anti-Thy-1.2 on POD 40 (Group 8, **Figure 1E**) was tested.

Table 3: Delayed PT/Cy on POD 30 – Alternative Approaches continued.

Group Number	Treatment	N
Group 6	Rapamycin + dPT/Cy +dBMT+ Anti-Thy1.2 (POD 40)	2
Group 7	Rapamycin + Fludarabine + dPT/Cy +dBMT+ Anti-Thy1.2 (POD 40)	6
Group 8	Rapamycin + 2*Fludarabine + dPT/Cy + 2*dBMT+ Anti-Thy1.2 (POD 40)	7

In these experiments we have primarily monitored allograft survival (**Figure 4A**). In Group 6, the repeated T cell depletion using anti-Thy-1.2 on POD 40 alone did not lead to durable mixed chimerism or long-term graft survival. In Group 7, fludarabine (POD 27 & 29) plus repeated T cell depletion using anti-Thy-1.2 on POD 40 promote graft survival with 50% of the animal demonstrating initial donor bone marrow engraftment. In Group 8, additional fludarabine (POD 25, 26, 27 & 28), additional dBMT and repeated T cell depletion using anti-Thy-1.2 on POD 40 further promote initial engraftment and chimerism levels. Donor-specific mixed chimerism analysis was performed on all animals enrolled in Group 6, 7 and 8. As shown in **Figure 4B**, hind limb recipients show an average of 5.33% (range: 0.33%-19.21%) of donor-specific chimerism in Group 6, 4.63% (range: 0.85%-18.97%) in Group 7 and 28.57% (range: 0.22%-57.04%) in Group 8.

Figure 4A: **Delayed PT/Cy on POD 30 – Alternative Approaches**. Allograft survival (4A) and donor-specific mixed chimerism (4B) in Groups 6, 7 and 8.



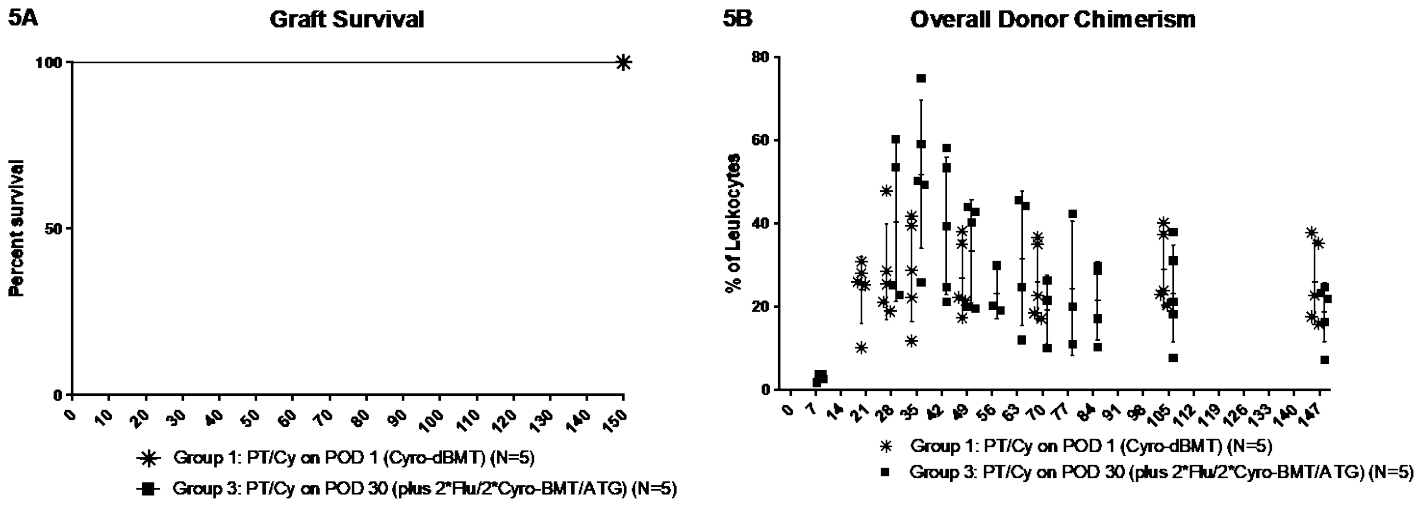
<u>Aim 1, Major Task 3</u>: Does fresh versus cryopreserved donor bone marrow affect the outcome of VCA allograft survival after delayed PTCy-based tolerance regimen.

<u>Subtask 1</u>: Compare the efficacy of cryopreserved donor bone marrow on VCA acceptance and stable mixed chimerism.

<u>Subtask 2</u>: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of PT/Cy.

Animals receiving a VCA under Major Task 3, Subtask 1 were receiving standard immunosuppression (Rapamycin 5 mg/kg) prior to enhanced delayed PT/Cy (dPT/Cy) treatment (**Figure 1A and 1E**). In addition, cryopreserved donor bone marrow transplantation (dBMT) is performed at the time of dPT/Cy on POD +1/+30, as demonstrated in **Figure 2**. During this reporting period 5 animals were added to Group 3. **Figure 5A** shows survival data for animals receiving dPT/Cy +/- dBMT on POD +1/+30. In Group 1, the graft survival of ongoing animals is 150 days, chimerism level is 25.87% (range 12.77%~32.63%). In Group 3 the graft survival of ongoing animals is 150 days, chimerism level is 35.67% (range 16.77%~30.15%) as shown in **Figure 5B**

Figure 5A: **Delayed PT/Cy on POD 30 – Alternative Approaches**. Allograft survival (5A) and donor-specific mixed chimerism (5B) in Groups 6, 7 and 8.



<u>Aim 2, Major Task 4:</u> Use Flow Cytometry, Computational Analysis, and high throughput sequencing to analyze the mechanisms of tolerance and the TCR repertoire in delayed tolerance induction via PT/Cy.

<u>Subtask 1:</u> Examine expression of exhaustion markers on T cells using flow cytometry.

During this reporting period, we continued to perform experiments via multi-color flow cytometry for analysis of T cell exhaustion using fluorochrome-labeled antibodies targeting cell surface antigens (**Table 4**). As shown in Figure **6A-C** we were able to obtain flow cytometry results from lymph node and spleen in animals surviving beyond 150 days with a functioning graft. To date, 5 of 8 (**Group 8**, (PT/Cy on POD30 plus 2*BMT), Aim 1, Major task 2, Subtask 1) were sacrificed for flow data acquisition. Among all the tested markers, PD-1 and Tim-3 expression on recipient $CD8^+$ T cells were found significantly increased in the graft marrow component (Figure **6B**, 24.4% +/- 14.46 in Group 8 versus 0.68% +/- 0.884% in naïve C57BL6 control animal).

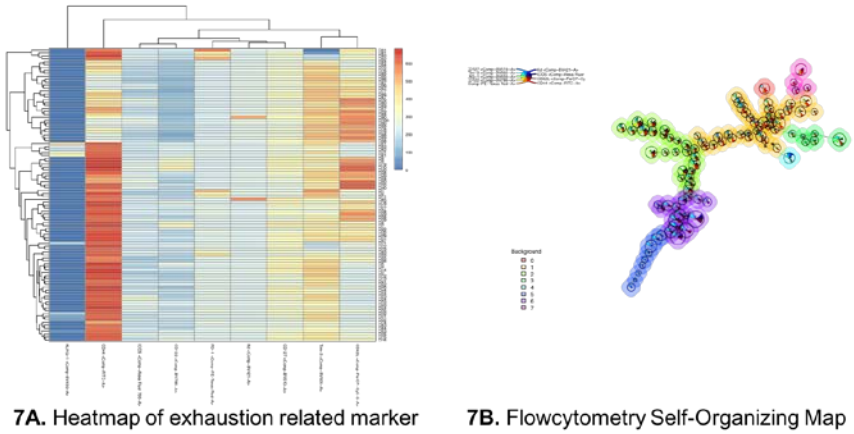
Table 4: Exhaustion Marker Flow Panel		
Marker	Fluorochrome	Clone
CD3	APC Cy7	145-2C11
CD4	PE Cy7	RM4-5
CD8	APC	53-6.7
Vb5.1/5.2	PE	MR-9
CD44	FITC	IM7
Kd	Pac Blue	SF1-1.1
CD16/32		2.4G2
CD62L	PerCP Cy5.5	MEL-14
PD1	PE Texas Red	J43
Tim3	BV605	5D12
KLRG1	BV650	2F1
CD127	BV510	A7R34
ICOS	AF700	C398.4A

Figure 6: Expression of T cell exhaustion markers PD-1 and TIM-3 in $CD3^{+}CD62L^{low}CD44^{high}KLRG^{negative}$ T cells in (A) spleen**,** (B) recipient bone marrow and (C) graft bone marrow.

<u>Aim 2, Major Task 4 – Subtask 2:</u> Perform computational flow cytometry analysis.

During this reporting period, we performed computational flow cytometer analysis using FlowSOM as shown in **Figure 7A and 7B.** Based on the results from ***Major Task 4 Subtask 1***, $CD3^{+}CD8^{+}$ T cells within the graft marrow need further analysis. Thus, graft bone marrow-derived $CD3^{+}CD8^{+}$ T cells were further selected for the remaining makers listed in **Table 4** and Mean Fluorescent Intensity (MFI) of each marker on all other channels were documented and compared. The heatmap in **Figure 7** shows compensated fluorochrome combinations representing biological marker combinations. Via analysis of these biomarkers we were able to demonstrate that limited subpopulations express exhaustion-related cell surface makers. However, certain alloreactive populations while expressing PD-1 do not express Tim-3. In addition, senescence was also found to play a role in the T cell dynamics. As shown by **Figure 7B**, by merging adjacent populations we are able to narrow down and identify 8 distinct populations, among which population 0 (with pink background) is bearing features of exhaustion ($KLRG\text{-}1^{-}PD\text{-}1^{+}Tim\text{-}3^{+}$) and will be the target of further investigation in the upcoming experiments.

Figure 7: Expression of T cell exhaustion related markers among naïve and tolerant animals.



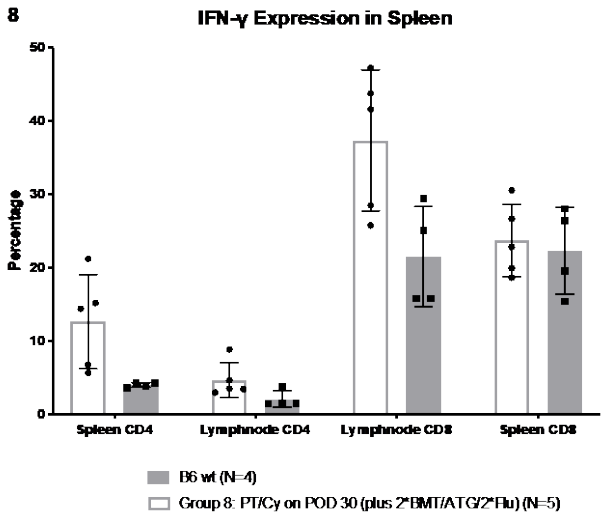
7A. Heatmap of exhaustion related marker **7B.** Flowcytometry Self-Organizing Map

<u>Aim 2, Major Task 4 - Subtask 3</u>: Conduct functional studies

We conducted functional experiments using *in-vitro* cytokine production assays to verify the functional status of splenocytes and lymphocytes in animals surviving beyond 150 days with a functioning graft. 5 Animals in Group 8 (PT/Cy on POD30 plus BMT, Aim 1, Major task 2, Subtask 1) were sacrificed to assess IFN-γ production as a marker

of T cell exhaustion, as shown in **Figure 8**. A significant elevation of IFN-γ was found in $CD4^+$ splenocytes (12.63% +/- 6.44 in Group 8 versus 3.98% +/- 0.304% in naïve C57BL6 control animals), but no significant differences were noted in other tissues when comparing WT to allograft recipients.

Figure 8: Cytokine Expression of T cell among naïve and tolerant animals.



Significant results or key outcomes

In our previous research, the use of PT/Cy in naïve untreated animals has shown powerful efficacy in inducing transplant tolerance and immunosuppression-free allograft survival. Results obtained during year one of this study demonstrated however that the use of delayed PT/Cy after transplantation in VCA recipients treated primarily with conventional immunosuppression does not lead to the same favorable outcome. Using alternative approaches, however, combining dPT/Cy with adjunct therapies, the investigators were able to successfully induce long-term immunosuppression-free allograft survival in this murine VCA transplant model. This accomplishment provides evidence that delayed tolerance induction is feasible in recipients of VCA treated chronically with conventional immunosuppression. The combination of optimal dosing of donor bone marrow, adjunctive therapy and VCA leads to chimerism induction and long-term immunosuppression-free allograft survival and will therefore contribute to a reduction in toxicity due to chronic immunosuppression. In-vitro findings indicated that donor specific T cell exhaustion may contribute to immune tolerance, however, this will need to be confirmed in additional mechanistic experiments over the next reporting periods. Overall, after several rounds of modifications and optimizations of the regimen, the investigators have developed a protocol that allows for robust delayed tolerance induction in an immunologically stringent small animal model. This will provide the foundation for translation of this regime to a pre-clinical large animal model as is proposed for the remainder of this project.

C. Training and Professional Development

Research performed under Aim 1 and 2 of the SOW has provided the PI's with the opportunity to teach critical thinking on how to apply current scientific and clinical knowledge to the development of a novel preclinical and translation tolerance induction protocol for VCA. Furthermore, these last three years served 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 T cell exhaustion.

D. Result Dissemination

Nothing to report

E. Future plan

During the upcoming Year of the performance period, we will focus on completion of experiments outlined in the SOW for Aim 3. In addition, we will conduct mechanistic studies outlined in Aim 2 completing the RNAseq experiments. These murine experiments will further lay the foundation for the start of the translational large animal studies in swine using the dPT/Cy approach.

4. IMPACT

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

Success in transplantation is limited by allograft survival and chronic host immunosuppression toxicity. This study aims to determine the feasibility of delayed induction of immune tolerance using a post-transplantation cyclophosphamide-based treatment protocol in patients currently on chronic immunosuppression to avoid the deleterious side effects of pharmacologic immunosuppression. Delayed transplantation tolerance will therefore provide practitioners with the opportunity to reduce long-term toxicity of immunosuppression by induction of transplant tolerance in patients who already received a VCA graft. In addition, however, if successful, this treatment concept may be expanded to a plethora of solid organ transplant recipients currently dependent on chronic immunosuppression. Furthermore, 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 by highlighting novel molecular and cellular mechanisms of delayed tolerance induction.

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 and bone marrow 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 factors have led to a delay in performance of *in-vivo* and *in vitro* experiments during Year 3 of the performance period and required the investigators to apply for a one-year no costs extension (NCE) for the project:

1. Requirement for development and testing of multiple alternative treatment approaches including dPT/Cy with adjunct therapy.
2. Development and optimization of routine multi-color flow cytometry for analysis of T cell exhaustion.
3. University wide research shut down between March and June due to Global COVID-19 pandemic.

A. Changes in Approach and Reasons for Change

As reported throughout the last reporting periods, the application of alternative treatment concepts including delayed PT/Cy with adjunctive treatment components (i.e: additional T cell depletion, additional donor bone marrow application as well fludarabine treatment) has allowed us to fully achieve objectives and goals outlined by the SOW. Using the now established enhanced delayed induction protocol (**Figure 1E**), we continue to perform experiment under Aim 2 Major Task 4. We have applied for a change of analysis of the TCR repertoire of donor and recipient T cells under the Aim 2 Subtask 4 to RNA sequencing of RNA encoding for exhaustion markers, e.g. TIM-3 and PD-1. When compared to TCR sequencing, RNA sequencing of exhaustion marker RNA could yield a more sophisticated description of the functional status of the T cell repertoire which would further help us understand the mechanism of immune regulation and long-term transplant tolerance in this murine VCA model.

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

With the ongoing pandemic of COVID-19, additional delays are anticipated in the upcoming reporting period.

C. Changes that had a Significant Impact on Expenditures

Currently focus is laid on anticipating future experiments and coordinating experimental plans, supplies and resources in order to allow a timely re-start of the *in-vivo* and *in-vitro* experiments.

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

Name: Gerald Brandacher, M.D.
Project Role: Principle Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Dr. Brandacher directly oversees the *in-vivo* and *in-vitro* experiments. Furthermore, he oversees reporting, budgeting and grantsmanship.

Name: Leo Luznik, M.D.
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Dr. Luznik is directly involved in the design, performance and analysis of *in-vitro* chimerism analysis and further contributes to the review and design of future experiments based on the data obtained during the last reporting period.

Name: Byoung Chol Oh
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: .24
Contribution to Project: Dr. Oh is directly involved in the design, performance and analysis of mechanistic *in-vitro* experiments and further contributes to the review and design of future experiments

Name: Yinan Guo, B.S.
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3
Contribution to Project: Mr. Guo performs all *in-vivo* and *in-vitro* experiments in collaboration with and under the supervision of the PI's. Furthermore, he contributes to data analysis and data presentation including writing of reports.

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

Gerald Brandacher	Change: Ended – W81XWH-16-C-0212 - Phase II: Novel Super-cooling of Genitourinary Cells and Tissues for Transplant Role: Site PI Effort: 1.08 CM Date: 10/01/2018 – 05/24/2020
Gerald Brandacher	Change: Ended – W81XWH-16-1-0708 - Engineering a Hybrid Thymus to Unravel the Tolerogenic Properties of Vascularize Role: Co-I Effort: .12 CM Date: 09/30/2016 – 06/29/2020

C. Other organizations involved as partners

Nothing to Report