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13. SUPPLEMENTARY NOTES

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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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. Plus, the use of living-related transplantation is ethically precluded in VCA. Hence, reconstructive transplantation is limited to cadaveric 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 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 the second year of this project, a total of n=80 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. Hindlimb 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**). As reported throughout the last reporting periods, however, the application of delayed PT/Cy alone did not fully achieve objectives and goals outlined by the SOW and thus alternative treatment concepts including delayed PT/Cy with adjunctive treatment components (i.e: additional T cell depletion, additional donor bone marrow transplantation and fludarabine treatment) were implemented within Year 2 of this project. **Figure 2** shows all tested alternative treatment concepts.

Figure 1: Delayed PT/Cy Treatment protocol. Figure **1A** shows the application of PTCy induction protocol with one day delay; Figure **1B** shows the application of PT/Cy induction protocol with 10 days delay; Figure **1C** shows the application of PT/Cy induction protocol with 30 days delay.

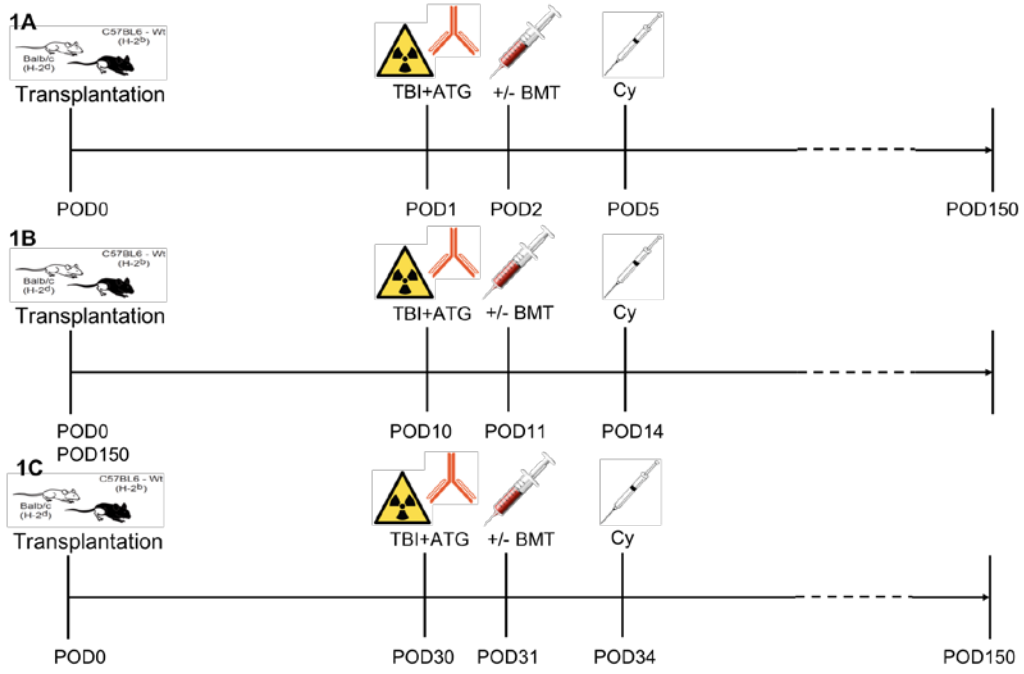
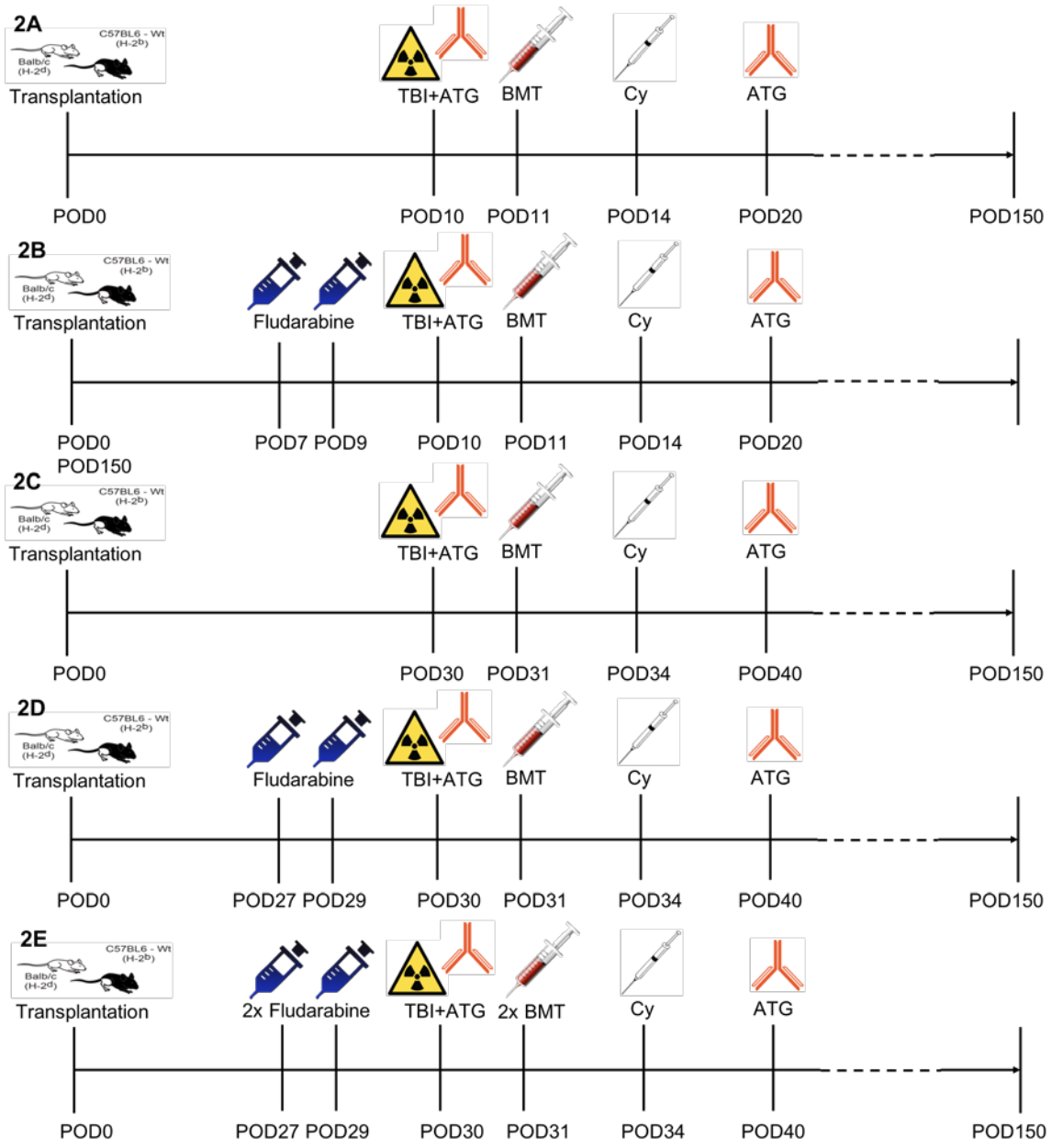


Figure 2: Alternative approaches - dPT/Cy with adjunctive therapy.

- **2A:** dPT/Cy on POD 10 with additional ATG on POD 20.
- **2B:** dPT/Cy on POD 10 with additional ATG on POD 20 and total 200mg/kg fludarabine on POD 7/9.
- **2C:** dPT/Cy on POD 30 with additional ATG on POD 40.
- **2D:** dPT/Cy on POD 30 with additional ATG on POD 40 and total 200 mg/kg fludarabine on POD 27/29.
- **2E:** 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.



A. Major Goals

The major goals of this project for Year 2 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.

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	95
Subtask 3: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of PT/Cy.	3-9	95
Major Task 2: Optimize delayed PTCy-based tolerance regimen that results in improved acceptance of VCA?	6-12	85
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	85
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	85
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	0
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	0
Subtask 2: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of PT/Cy.	9-12	0

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.		
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	25
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	25
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	0
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	0
Milestone #2: Manuscript on delayed PTCy-based tolerance regimen in a translational rodent VCA model and associated mechanisms	18-24	

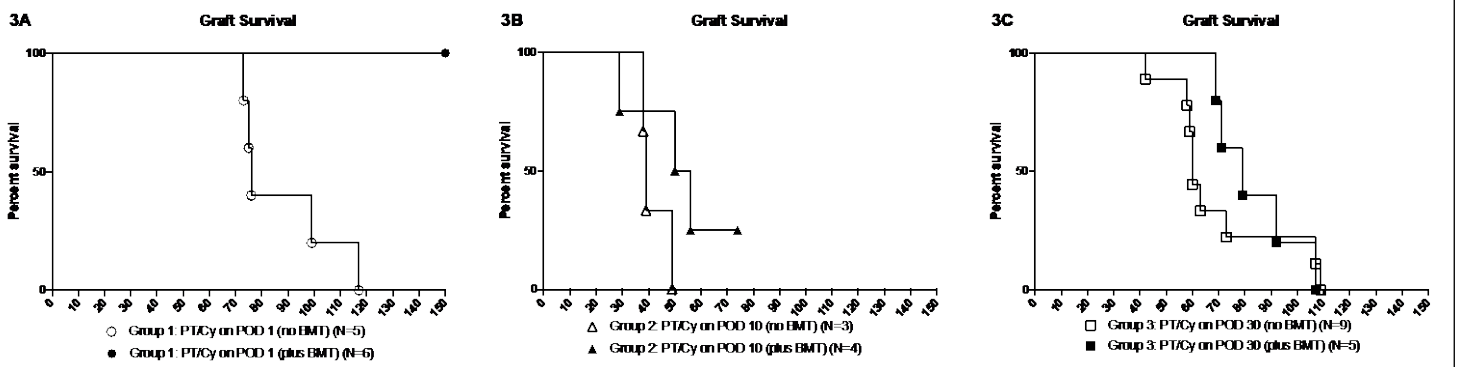
B. Accomplishment of Goals

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?

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:

All experimental animals enrolled in this Major Task during Year 1 and 2 of the performance period received orthotopic hind limb transplantation across a full MHC mismatch. Recipient animals were treated with Rapamycin 5 mg/kg for either 1 day (Group 1), 10 days (Group 2) or 30 days (Group 3) prior to the application of delayed PT/Cy (dPT/Cy). Select animals received additional donor bone marrow transplantation (dBMT) with dPT/Cy application. As shown in **Figure 3**, animals receiving dPT/Cy after 1 day of conventional immunosuppression demonstrate prolonged allograft survival while the application of dPT/Cy after 10 or 30 days of rapamycin treatment does not lead to extended graft survival. The addition of dBMT, however, lead to indefinite allograft survival when combined with dPT/Cy early on POD +1 (**Figure 3A**) yet did not influence allograft survival for groups with more delayed induction treatment (**Figure 3B-3C**).

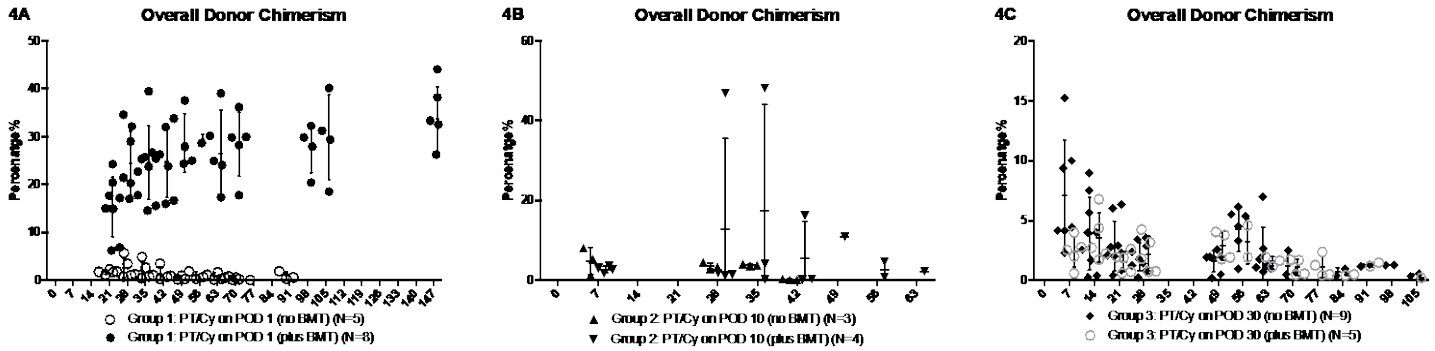
Figure 3: Application of delayed PTCy induction protocol with or without additional donor bone marrow in Group 1-3. The combination of donor bone marrow and dPT/Cy leads to indefinite animal and graft surival if given on POD +1 **(3A),** however, allograft survival is not proglonged when dBMT and dPT/Cy are applied on POD +10 **(3B)** or +30 **(3C).**



Subtask 3: Evaluate the degree and durability of chimerism after VCA in Groups 1-3 after the application of dPT/Cy:

All animals enrolled in Subtask 2 receive continued flow cytometry-based donor-specific mixed chimerism analysis under Subtask 3. As shown by **Figure 4**, hind limb recipients show an average of 23.80% (range: 6.21%-39.49%) of donor-specific chimerism in Group 1 (**Figure 4A**), 8.88% (range: 0.16%-48.16%) in Group 2 (**Figure 4B**); 2.13% (range: 0.03%-6.78%) in Group 3 (**Figure 4C**) throughout the course of allograft survival.

Figure 4: Donor-specific mixed chimerism in Group 1-3.



In summary, data obtained up to date demonstrates that the application of delayed PT/Cy at various time points (i.e. POD 1, 10, 30) after VCA leads to prolonged allograft survival compared to untreated control animals (MST 8 days, data not shown). The addition of dBMT on POD 1 (Group 1 + dBMT) induces high levels of donor-specific mixed chimerism (**Figure 4A**) and promotes immunosuppression-free VCA survival (see above: **Figure 3A**). However, neither treatment with dPT/Cy alone or dPT/Cy combined with dBMT induces immunological tolerance and indefinite VCA survival if applied with increased delay (i.e. POD 10 or 30).

Aim 1, Major Task 1: Alternative Treatment Approaches.

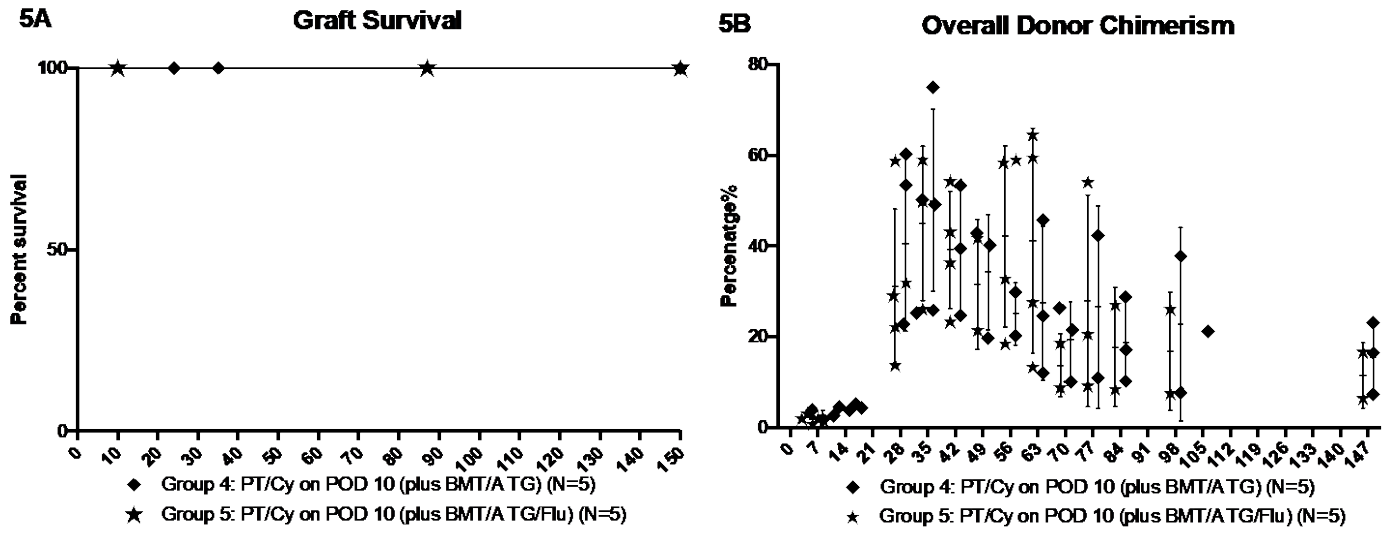
Therefore, in an attempt to achieve the primary objective of immunosuppression-free VCA survival and tolerance by dPT/Cy application we further modified the treatment protocol by employing "alternative approaches" outlined by the grant narrative (see introduction and **Figure 2** above and **Table 2** below). These include the combination of dPT/Cy with adjunctive treatment components (i.e.: additional T cell depletion, additional dBMT and fludarabine treatment).

Table 2: Delayed PT/Cy on POD 10 – Alternative Approaches.

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

As demonstrated by data in **Figure 5**, on POD 10 the combination of dPT/Cy and dBMT with T cell depletion alone or additional fludarabine and T cell depletion significantly improved both allograft survival and chimerism levels. 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 5: Delayed PT/Cy on POD 10 – Alternative Approaches. Allograft survival (**5A**) and donor-specific mixed chimerism (**5B**) in Groups 4 and 5.



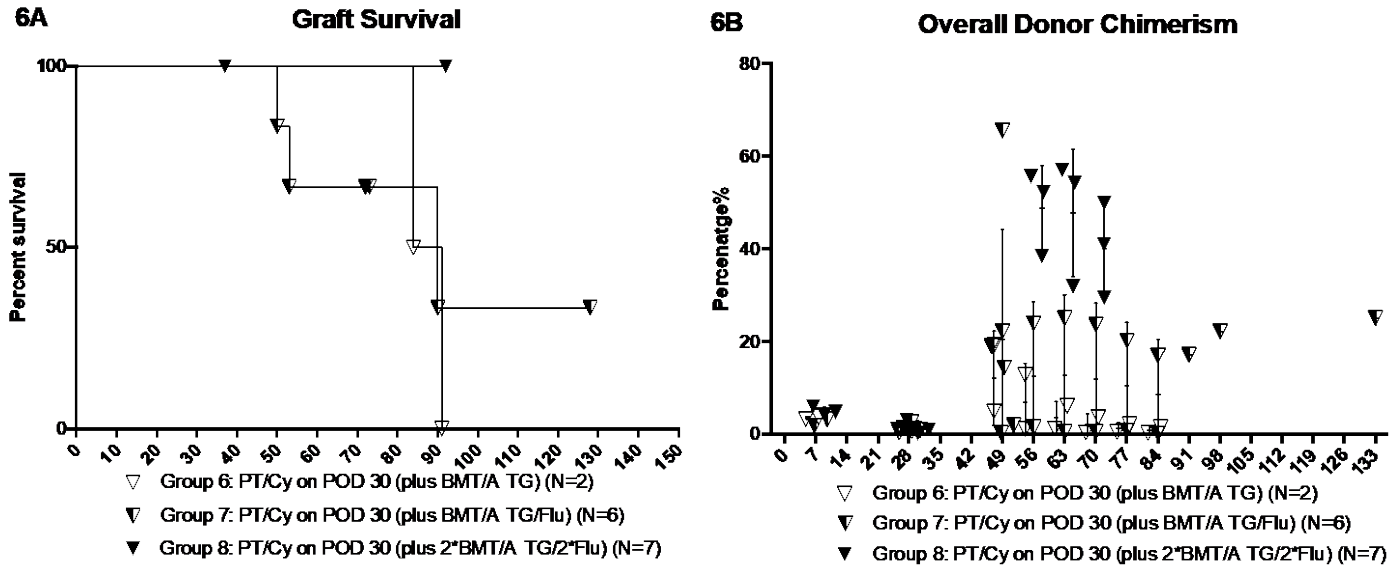
The results using adjunct therapy on POD 10 encouraged us to further apply this treatment 30 days after transplantation (Groups 3/POD 30). As shown by **Table 3** and illustrated by **Figure 2** repeated T cell depletion using anti-Thy-1.2 alone on POD 40 (Group 6, **Figure 2C**); fludarabine (POD 27 & 29) and repeated T cell depletion using anti-Thy-1.2 on POD 40 (Group 7, **Figure 2D**) and additional doses of fludarabine (POD 25, 26, 27 & 28), an additional dose of donor bone marrow and repeated T cell depletion using anti-Thy-1.2 on POD 40 (Group 8, **Figure 2E**) 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
* additional dose		

In these ongoing experiments we are currently monitoring graft survival (**Figure 6A**). 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 is performed on all animals enrolled in Group 6, 7 and 8. As outlined by **Figure 6B**, 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 6A: **Delayed PT/Cy on POD 30 – Alternative Approaches**. Allograft survival (5A) and donor-specific mixed chimerism (5B) in Groups 6, 7 and 8.



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.

Subtask 1: Examine expression of exhaustion markers on T cells using flow cytometry.

In Year 2, we performed experiments to examine expression of exhaustion markers on T cells using fluorochrome-labeled antibodies targeting cell surface antigens as listed in **Table 4** from peripheral blood, lymphnode, spleen and bone marrow in animals with long-term graft survival. As shown in **Figure 7**, the expression of markers of T cell exhaustion is found to be up-regulated in animals with long-term immunosuppression-free allograft survival suggesting a possible role for T cell exhaustion as part of the mechanism of long-term graft acceptance.

Table 4: Exhaustion Marker 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 7A: Exemplary expression of T cells exhaustion markers in bone marrow.

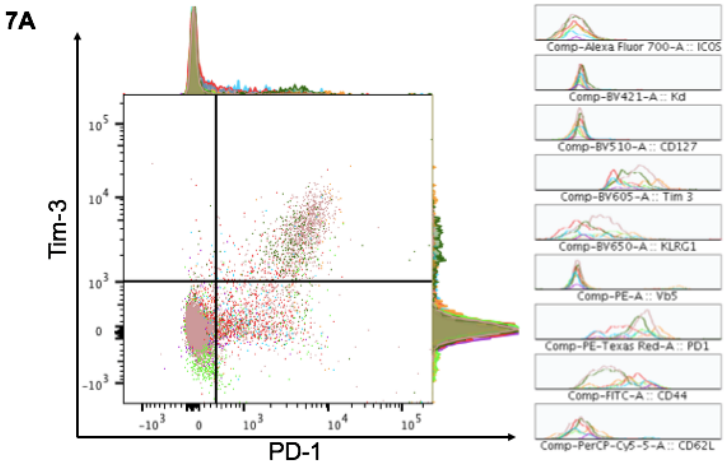
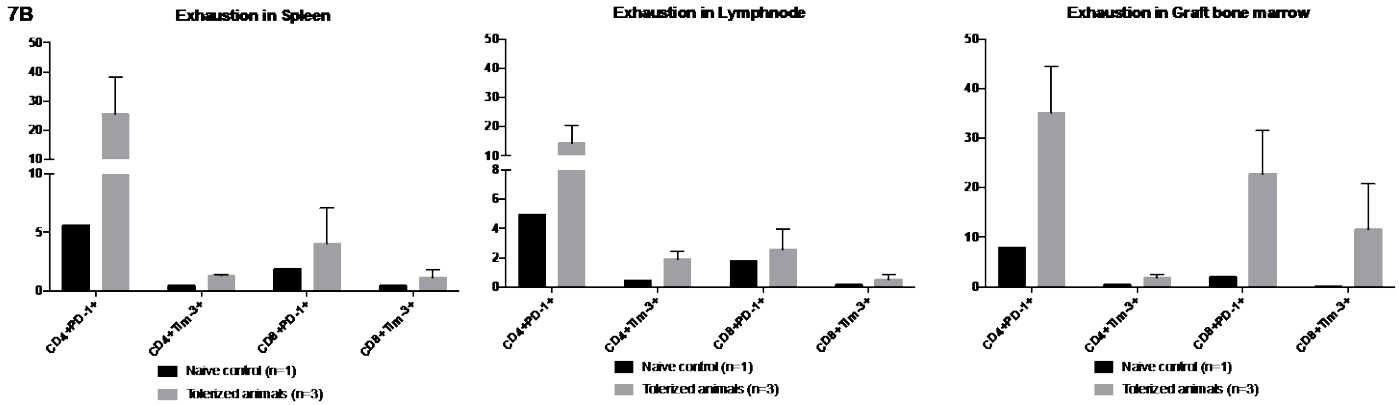


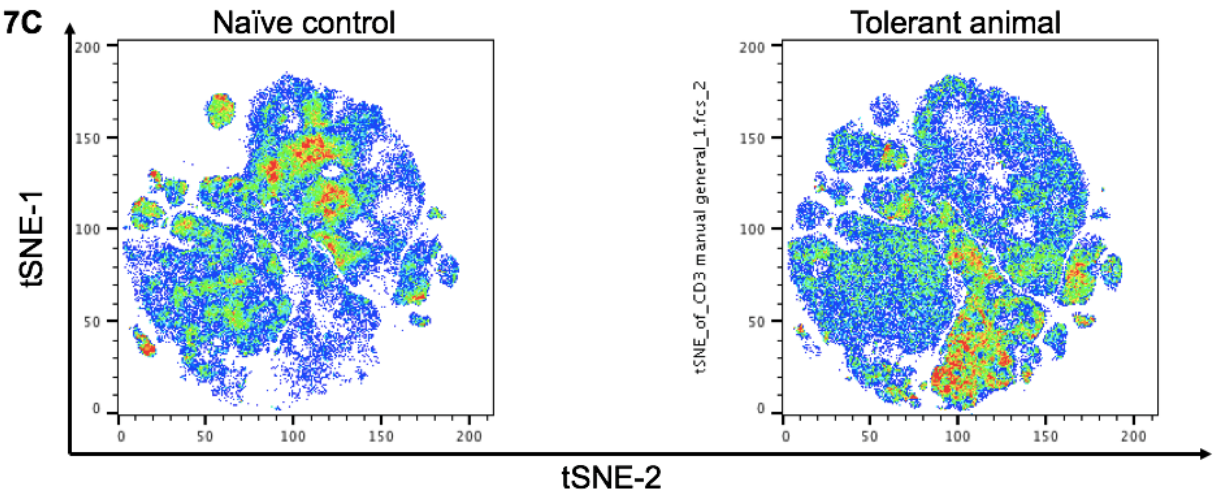
Figure 7B: Expression of T cell exhaustionmarkers among different tissue.



Aim 2, Major Task 4 – Subtask 2: Perform computational flow cytometry analysis.

Computational flow cytometry analysis (viSNE) allows for the visualization of high-dimensional data on a single bivariate plot, which, in our case, reduces a 12-dimensional data space into 2 dimensions while still maintaining the structure of the data. The expression of exhaustion markers as outlined in **Table 4** were translated into a 2-D geographical map (**Figure 7C**), which would allow us to compare all the different populations at the same rather than using single bivariate plots for each fluorochrome combination as displayed in the histogram in **Figure 7A.**

Figure 7C: Expression of T cell exhaustion related markers 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 induced 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.

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 two 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 memory T cell analysis.

D. Result Dissemination

Nothing to report

E. Future plan

During Year 3 of the performance period, we will focus on completion of experiments outlined in the SOW for Aim 1. In addition, we will conduct mechanistic studies outlined in Aim 2 using the optimized alternative treatment approaches. 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 two factors have led to a delay in performance of *in-vivo* and *in vitro* experiments during Year 2 of the performance period:

1. Development and testing of alternative approaches including dPT/Cy with adjunct therapy.
2. Development of routine routine multi-color flow cytometry for analysis of T cell exhaustion.

A. Changes in Approach and Reasons for Change

As reported throughout the last reporting periods, the application of delayed PT/Cy alone did not fully achieve objectives and goals outlined by the SOW and thus 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) were successfully implemented within Year 2 of this project. While final survival data will still mature over the next reporting periods we will use one of the tested alternative protocols to conduct studies outlined in Aim 2 of the SOW.

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

Determination of the most optimal treatment adjunct to the dPT/Cy protocol to promote allograft survival have added additional time to complete Aim 1 and 2. Once the most optimal treatment concepts have matured we will be able to finalize experiments delayed in Year 2 during this upcoming performance period of Year 3.

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