

AWARD NUMBER: W81XWH-13-2-0060

TITLE: The Johns Hopkins RTR Consortium: A Collaborative Approach to Advance Translational Science and Standardize Clinical Monitoring of Restorative Transplantation

PRINCIPAL INVESTIGATOR: Gerald Brandacher, MD

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205-1832

REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE*Form Approved*
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE October 2016	2. REPORT TYPE Annual	3. DATES COVERED 09/15/2015 – 09/14/2016
4. TITLE AND SUBTITLE The Johns Hopkins RTR Consortium: A Collaborative Approach to Advance Translational Science and Standardize Clinical Monitoring of Restorative Transplantation		5a. CONTRACT NUMBER
		5b. GRANT NUMBER W81XWH-13-2-0060
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Gerald Brandacher 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 733 N. Broadway Suite 117 Baltimore, MD 21205		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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			
<p>For many devastating combat and civilian injuries where conventional reconstruction is inadequate, vascularized composite allotransplantation (VCA) has become a viable alternative. However, the toxicities and adverse effects of high dose immunosuppressive drugs have curtailed wider application. Thus the purpose of this project is to develop novel clinically relevant immunosuppression sparing regimens allowing for immunomodulation and tolerance induction after VCA using a translational large animal model. A total of 24 MGH miniature swine underwent heterotopic osteomyocutaneous hind limb transplantation across full swine leukocyte antigen mismatch. All animals received non-myeloablative conditioning with 50cGy total body and 350cGy thymic irradiation for induction. Aim1: Group I was treated with high-dose tacrolimus (15-20ng/ml) maintenance therapy. Group II was treated with low-dose tacrolimus (4-6ng/ml). Group III received low-dose tacrolimus and 20 mg/kg of CTLA4-Ig administered on POD2, 7, 14, 30, 60, 90, and 120. Aim2: Group IV received transient high-dose tacrolimus until POD60. Group V received transient high-dose tacrolimus until POD60 and was switched to CTLA4-Ig administered on POD60, 85, 100, 120 and 150. Aim3: Group VI received the non-myeloablative conditioning plus bone marrow infusion (BMI) and intermediate dose tacrolimus (10-15 ng/ml) for 30 days only. Group VIII received the induction regimen, BMI and CTLA4-Ig and a short-term dose of tacrolimus (30 days). In all groups, graft rejection was monitored by clinical assessment and protocol skin biopsies. Alloreactivity against donor antigens was assessed using an optimized CFSE-based mixed lymphocyte reaction (MLR). All group I animals died prematurely due to infectious complications related to high dose tacrolimus treatment. 2/3 animals that received sub-therapeutic tacrolimus (group II) have rejected their grafts. 3/5 animals that received belatacept in addition to low dose tacrolimus (group III) have achieved long-term graft survival (>230 days). In the current reporting period (Aim 2 and Aim 3), 3/3 animals in group IV and 4/5 animals in Group V achieved indefinite graft survival (beyond POD300) despite weaning of all immunosuppression. The one animal in Group V that rejected its graft began to show evidence of late rejection on POD277. During Aim 3, a total of 6 animals were transplanted in Group VI. Three animals in this group were euthanized before day 30 due to complications (bleeding, hematoma, and post-transplant lymphoproliferative disorder). Two animals in Group VI display long-term graft survival (currently over 320 days), while one animal displayed signs of mechanical trauma approximately around POD 300, triggering loss of the whole graft by POD365. Donor specific unresponsiveness was confirmed in all long-term survivors <i>in vitro</i> by CFSE-MLR. Two animals were transplanted in Group VIII. Both animals completed their course of CTLA4-Ig and were maintained on high-dose tacrolimus after transplant but developed lethargy, anorexia, neutropenia and respiratory distress and were euthanized before POD20. Pathology findings have identified radiation-related cardiac injury as a potential contributor to their clinical outcomes.</p> <p>Overall, our results indicate that the addition of CTLA4-Ig to subtherapeutic CNI does not appear to be able to prevent graft rejection. Furthermore, tolerance of VCA containing a vascularized bone component may be achieved with a conditioning regimen of non-myeloablative irradiation and peritransplant tacrolimus. The long-term graft survival off of immunosuppression for animals treated with low dose or high-dose tacrolimus suggest that the vascularized bone component of the composite graft may have a more robust immunomodulatory effect than expected. Additional clinical follow-up and <i>in vitro</i> experiments are ongoing to mechanistically characterize the immune status of the current recipients.</p>			
15. SUBJECT TERMS			
Vascularized composite allotransplantation (VCA), Hand transplantation, Face transplantation, Tolerance induction, Immunomodulation, Chimerism, Costimulatory blockade, Belatacept, CTLA4-Ig, Large animal model			
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified
			18. NUMBER OF PAGES 24
			19a. NAME OF RESPONSIBLE PERSON USAMRMC
			19b. TELEPHONE NUMBER <i>(include area code)</i>

TABLE OF CONTENTS

1. INTRODUCTION	2
2. KEYWORDS	3
3. ACCOMPLISHMENTS	3
a. Major Goals	
b. Accomplishments Under these Goals	
c. Opportunities for Training and Professional Development	
d. Dissemination of Results	
e. Plans for Next Reporting Period	
4. IMPACT	11
a. Impact on the Development of the Principal Discipline(s) of the Project	
b. Impact on Technology Transfer	
c. Impact on Society Beyond Science and Technology	
5. CHANGES/PROBLEMS	12
a. Changes in Approach	
b. Problems/Delays and Plans for Resolution	
c. Changes that Impacted Expenditures	
d. Changes in use or care of vertebrate animals, biohazards, and/or select agents	
6. PRODUCTS	13
a. Publications	
b. Websites	
c. Technology or Techniques	
d. Inventions	
e. Other Products	
7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS	13
a. Individuals	
b. Changes in Active Other Support of PI	
c. Partner Organizations	
8. SPECIAL REPORTING REQUIREMENTS	14
a. Collaborative Awards	
b. Quad Charts	
9. APPENDICES	14

1. INTRODUCTION

Close to 40% of combat injuries sustained in Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) involved severe extremity or craniofacial trauma. Currently, despite the best reconstructive efforts using native tissue, these injuries are not only mutilating, but frequently result in permanent disfigurement and morbidity. For many devastating combat and civilian injuries where conventional reconstruction is inadequate, vascularized composite allotransplantation (VCA) has become a viable alternative. However, the toxicities and adverse effects of the high dose immunosuppressive drugs have curtailed wider application. In particular, the use of calcineurin inhibitors (CNIs, i.e. tacrolimus), currently the mainstay therapy in VCA, is associated with substantial morbidity and is relatively ineffective in preventing antibody mediated injury and chronic rejection.

Thus, the central challenge for VCA is to develop novel treatment concepts to minimize/avoid immunosuppression and extend the benefits of these life-enhancing procedures to the military and civilian patient populations. Biologic agents such as the monoclonal antibody Cytotoxic T-lymphocyte-associated antigen-4 immunoglobulin (CTLA4-Ig) (e.g., abatacept, belatacept), which block T-cell costimulation, have been developed to overcome this limitation, and represent a new paradigm in immunosuppression - biological therapy for maintenance immunosuppression devoid of the toxicities associated with CNIs.

In this study we propose to use the second-generation, FDA approved, CTLA4-Ig belatacept, that has demonstrated potent inhibition of T-cell activation and proven effective in phase II and III trials of kidney transplantation, to develop clinically relevant regimens for immunomodulation and tolerance induction after VCA using a translational large animal model.

2. KEYWORDS

Vascularized Composite allotransplantation (VCA)
Hand transplantation
Face transplantation
Tolerance induction
Immunomodulation
Chimerism
Costimulatory blockade
Belatacept
CTLA4-Ig
Large Animal model

3. ACCOMPLISHMENTS

a. What were the major goals of the project?

The major goals of this project for year 1-3 of this project as outlined by the approved statement of work are:

Phase 1, Aim 1: Establish a belatacept-based protocol to enable CNI minimization after VCA.

TASK 1. Obtain institutional Animal Care and Use Committee (ACUC) approval.

TASK 2. Obtain DoD Animal Care and Use Review Office (ACURO) approval

TASK 3. Adapt clinically established induction and CNI maintenance regimen in a fully SLA-mismatched swine hind limb transplantation model.

SUBTASK 1. Perform hind limb transplantation with high-dose TAC maintenance therapy (Group I; n=3)

SUBTASK 2. Perform hind limb transplantation with sub-therapeutic, low-dose TAC treatment (Group II; n=3)

TASK 4. Determine impact of peritransplant belatacept treatment to allow for allograft survival with low-dose (sub-therapeutic) CNI treatment.

SUBTASK 1. Perform hind limb transplantation with low-dose TAC in combination with belatacept (Group III; n=5)

Phase 2, Aim 2: Investigate the possibility to convert from conventional CNI-based immunosuppression to belatacept maintenance with subsequent CNI withdrawal.

TASK 1. Attempt CNI weaning/withdrawal without CTLA4-Ig and assess time course of allograft rejection.

SUBTASK 1. Perform hind limb transplantation with high-dose TAC (60 days) + subsequent TAC weaning (Group IV; n=3)

TASK 2. Repeat CNI weaning/withdrawal protocol with delayed belatacept treatment and maintenance

SUBTASK 1. Perform hind limb transplantation with high-dose TAC (60 days) + late CTLA4-Ig + subsequent TAC weaning and CTLA4-Ig maintenance (Group V; n=5)

Phase 2, Aim 3: To compare immunomodulatory donor BM infusion to BM transplantation with establishment of durable mixed chimerism for induction tolerance and/or VCA survival on CNI free immunosuppression using a belatacept-based regimen.

TASK 1. Evaluate the effects of donor BMI augmentation (JHH) vs. BMT (MGH)

SUBTASK 1. Perform hind limb transplantation with BMI protocol + short term TAC (30 days), Group VI; n=6)

TASK 2. To test tolerogenic effects of combined BMI(JHH)/BMT(MGH) and peri-transplant CTLA4-Ig

SUBTASK 1. Perform hind limb transplantation with BMI protocol + short term TAC (30 days) + Perioperative Belatacept (Group VIII; n=2)

b. What was accomplished under these goals?

Phase 1, Aim 1, Year 1: Establish a belatacept-based protocol to enable CNI minimization after VCA.

Aim 1, Tasks 1: Obtain institutional Animal Care and Use Committee (ACUC) approval

Status: Complete; 100%

The specific objectives of this task were met by the approval of the IACUC protocols, allowing the VCA Laboratory to perform the proposed *in-vivo* transplantation experiments.

Aim 1, Task 2: Obtain DoD Animal Care and Use Review Office (ACURO) approval

Status: Complete; 100%

The specific objectives of this task were met by the approval of the ACURO protocols, allowing the VCA Laboratory to perform the proposed *in-vivo* transplantation experiments.

Aim 1, Task 3: Adapt clinically established induction and CNI maintenance regimen in a fully SLA-mismatched swine hind limb transplantation model

Status: Complete; 100%

In Phase 1 of the study eleven swine heterotopic hind limb transplants were performed across a full swine leukocyte antigen (SLA-) barrier and animals were enrolled in group 1-3 as outlined by the approved statement of work (SOW) (Table 1). Data acquisition and tissue sampling is complete.

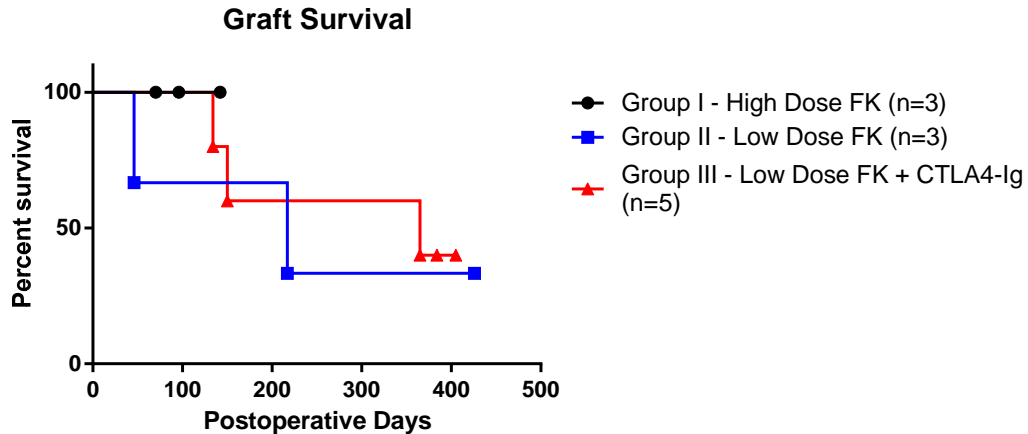
Table 1: Aim 1 - Experimental Groups

Group	N	SLA Mismatch	Protocol	Rational	Status
I	3	Full	Induction + high-dose TAC maintenance	Control Group: therapeutic CNI	100%
II	3	Full	Induction + low-dose TAC maintenance	Control Group: sub-therapeutic CNI	95%
III	5	Full	Induction + low-dose TAC + CTLA4-Ig maintenance	Experimental Group: Tests role of CTLA4-Ig to allow for graft survival with minimal CNI	95%

Aim 1, Task 3, Subtask 1: Perform hind limb transplantation with high-dose TAC maintenance therapy (Group I; n=3)

All animals in Group 1 (n=3) have undergone heterotopic hind limb allotransplantation. As hypothesized, high dose tacrolimus (trough level 10-20 ng/mL) has maintained all allografts without clinical signs of rejection throughout the study period (Figure 1). However, in attempting to maintain high dose tacrolimus, it was not uncommon for levels to reach significantly higher trough levels (i.e. 40-60 ng/mL). All three animals died prematurely due to infectious complications associated with high dose immunosuppression. Pig 22312 experienced sudden respiratory arrest and was found to have an airway obstruction from a hyperplastic pharyngeal lymph node. On necropsy, generalized lymphadenopathy was found throughout the animal, including mediastinal and mesenteric lymph nodes. A neutrophilic and histiocytic predominance in the lymph nodes points more towards infection rather than post-transplant lymphoproliferative disease (PTLD). The second premature death in this group, Pig 22227, was due to a gastrointestinal bleed, related to either infectious gastroenteritis/colitis or stress ulcer formation. The third animal, Pig 22229, was euthanized at POD 142 due to infection as well (Figure 1).

Figure 1: Animal and graft survival for animals from subtasks 1.3.1 (Group I, Black), 1.3.2 (Group II, Blue), 1.4.1 (Group III, Red). Note that Group I animals experienced premature deaths due to complications while maintaining allografts without rejection. Group II and III have not experienced premature deaths as tacrolimus levels are kept in a sub-therapeutic range. 2/3 animals in Groups II have rejected their allograft, whereas 3/5 animals in group III have maintained their graft rejection-free long term off of all immunosuppression.



Overall, Group 1 has demonstrated viable heterotopic hind limb allografts while receiving high dose tacrolimus but all three animals died prematurely due to complications associated with significant immunosuppression. These findings support our hypothesis that high dose tacrolimus can maintain vascularized composite allotransplants, but does so at the cost of significant complications.

1.3.2 Aim 1, Task 3, Subtask 2: Perform hind limb transplantation with sub-therapeutic, low-dose TAC treatment (Group II; n=3)

All animals in Group 2 (n=3) have undergone heterotopic hind limb allotransplantation and were maintained on low dose tacrolimus treatment (trough levels 4-6 ng/mL). Pig 22309, upon lowering the dose of tacrolimus to the target range of 4-6 ng/mL, briskly rejected the graft within 20 days on POD 46 (Figure 2). The first animal from Group 2 demonstrated the inability of low dose tacrolimus (4-6 ng/mL) to maintain the allotransplant and prevent rejection. Pig 22573 did not demonstrate overt evidence of rejection until POD 191 and was euthanized at POD 217 when it reached grade IV rejection. Interestingly, Pig 22545 has achieved long term graft survival with only low dose tacrolimus immunosuppression for 150 days post-transplant (Figure 3). This observation was unexpected and may be explained by the immunomodulatory effect of the transplanted donor bone marrow niche carried by the vascularized bone component of the composite graft. Pig 22545, the last ongoing animal was ultimately euthanized on POD 487 with a perfectly normal graft without any signs of rejection (Figure 1). Samples have been collected for planned chimerism, MLRs and histopathological studies which are currently ongoing in collaboration with the group at MGH..

Figure 2. Clinical and histologic images of an example of graft rejection in pig 22309 on POD 46. A.) Clinical image of complete epidermal necrosis. B.) Histologic image of skin biopsy from the graft demonstrating severe mononuclear cell infiltrate with loss of epidermal and dermal junction. C.) Muscle biopsy showing

minimal inflammatory infiltrates indicating that the strong reactive response against skin is not present in other parts of the composite allograft.

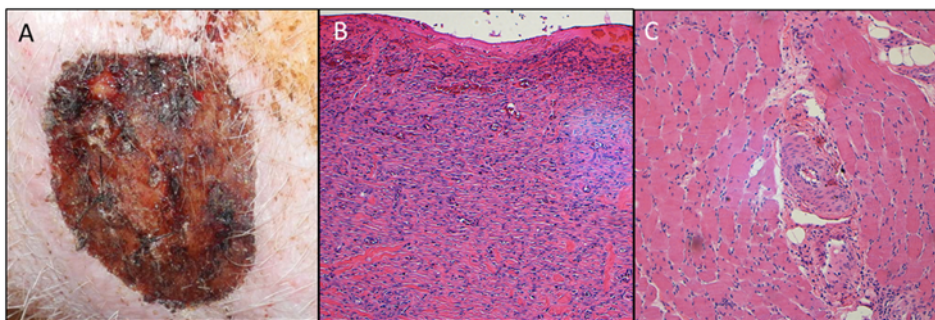
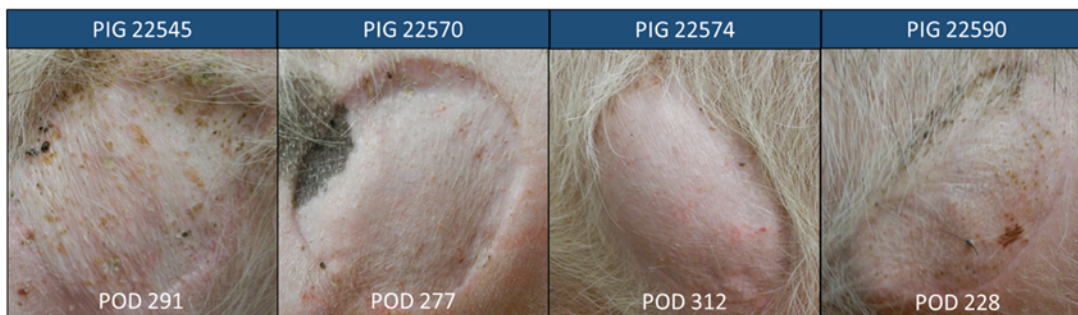


Figure 3: Clinical images of allografts from long-term graft survival animals in subtask 1.3.2 and 1.4.1. Pig 22545 is in group II and has received low dose tacrolimus until POD 150 and currently has no signs of rejection while being off of all immunosuppression. Pig 22570, 22575 and 22590 received belatacept in addition to low dose tacrolimus until POD 150 and have all achieved long-term rejection free graft survival.



Aim 1, Task 4: Determine impact of peritransplant belatacept treatment to allow for allograft survival with low-dose (sub-therapeutic) CNI treatment

Aim 1, Task 4, Subtask 1: Perform hind limb transplantation with low-dose TAC in combination with belatacept (Group III; n=5)

All animals in Group 3 (n=5) have undergone heterotopic hind limb allotransplantation and were maintained on low dose tacrolimus treatment (4-6 ng/mL) along with intermittent belatacept infusion administered on POD 2, 7, 14, 30, 60, 90, 120, 150. Two animals (Pig 22575 and 22571) rejected their graft at POD 150 and POD 134, respectively (Figure 1). The other three animals in this group all achieved long-term rejection-free graft survival despite cessation of all immunosuppressive agents by POD150 (Figure 3). Long-term surviving pigs 22590 and 22574 were ultimately euthanized on POD 445 and 508, respectively, with no evidence of graft rejection (Figure 1). Final chimerism analysis on these animals is currently ongoing.

These results are very encouraging and suggest the potential efficacy of our induction regimen with belatacept in conjunction with low dose tacrolimus, thereby avoiding the

complications of high dose CNI. Long-term survival of all components of the vascularized composite allograft has not been previously demonstrated with this regimen in a large animal model.

Further mechanistic studies are underway to attempt to elucidate the role of the donor vascularized bone marrow niche present from the transplanted lower extremity long bones.

Phase 2, Aim 2: Investigate the possibility to convert from conventional CNI-based immunosuppression to belatacept maintenance with subsequent CNI withdrawal.

Status: Incomplete; 95%

In Phase 2 of the study eight swine heterotopic hind limb transplants were performed across a full swine leukocyte antigen (SLA-) barrier and animals were enrolled in group 4 & 5 as outlined by the approved statement of work (SOW) (Table 2). Final chimerism analysis for this phase of the study is still ongoing in collaboration with the group at MGH..

Table 2: Aim 2 – Experimental Groups

Group	N	SLA Mismatch	Protocol	Rational	Status
IV	3	Full	Induction + high-dose TAC (60 days) + Subsequent TAC weaning	Control Group: CNI weaning without CTLA4-Ig	95%
V	5	Full	Induction + high-dose TAC (60 days) + late CTLA4-Ig + Subsequent TAC weaning and CTLA4-Ig maintenance	Experimental Group: Investigate the ability to wean/withdraw CNIs after delayed CTLA4-Ig treatment and maintenance	95%

2.1 Aim 2, Task 1: Attempt CNI weaning/withdrawal without CTLA4-Ig and assess time course of allograft rejection.

All animals in group IV (n=3) have undergone heterotopic hind limb allotransplantation. Pigs 22753, 22755 and 22757 were euthanized on POD 396, 382 and 381, respectively. There was no evidence of graft rejection while all three animals have been off of immunosuppression since POD 60 (Figure 4). Samples have been collected for planned chimerism studies. MLRs have been performed on all three animals and confirm presence of donor specific unresponsiveness. A representative comprehensive clinical, histological

and in immunological sample is shown in Figure 5 and all clinical grafts for the three animals are shown in Figure 6.

Most strikingly and in sharp contrast to group I animals that received high dose tacrolimus for 150 days and were complicated by significant infectious complications, group IV animals have not demonstrated significant toxicity from their peritransplant tacrolimus therapy. This may be in part explained by the shorter course of therapy and the improved maintenance of tacrolimus trough within the target range of (10-20 ng/mL) (Figure 7). Overall, contrary to our initial hypothesis, group IV animals have not rejected their allograft soon after weaning of CNI. These findings correlate well with our previous findings that the vascularized bone marrow component contained within the VCA may exert potent immunomodulatory effects. Final mechanistic studies to elucidate the underlying immunoregulatory mechanisms in this group are still ongoing.

Figure 4. Graft survival curves for animals from subtasks 2.1.1 (Group IV, Orange) and 2.2.1 (Group V, Green): high dose tacrolimus (15-20 ng/mL) for 60 days ceased or transitioned to intermittent belatacept, respectively. Note none of the animal have died prematurely or rejected (Grade IV) their grafts.

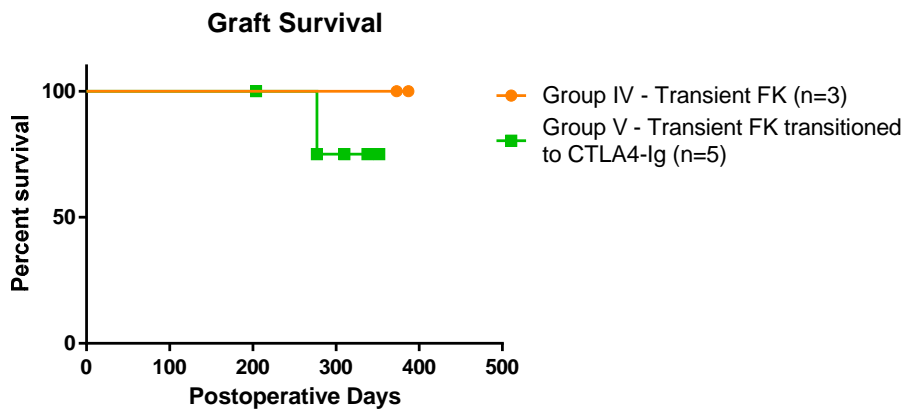


Figure 5. Graft appearance, histology and mixed lymphocyte reaction (MLR) of a representative animal confirming long-term graft survival and presence of donor specific unresponsiveness. Representative data from Pig 22753 (Group IV) demonstrating evidence of long term graft survival and donor-specific non-responsiveness. Clinical image and H&E stain of skin biopsy sample taken on POD253 demonstrate no gross or histologic evidence of graft rejection. MLR of T cell subsets (CD4+ and CD8+) from Pig 22753 PBMC compared to naïve GG pig control. 3rd party stimulation was by PBMC from a DD animal. Both Pig 22753 and naïve GG control animal has robust lymphocyte proliferative response to 3rd party stimulation, however, Pig 22753 has minimal response to PBMC from its donor whereas donor PBMC elicits a strong response in naïve GG animal similar to that with 3rd party stimulation. Similar results were seen with 22755 and 22757.

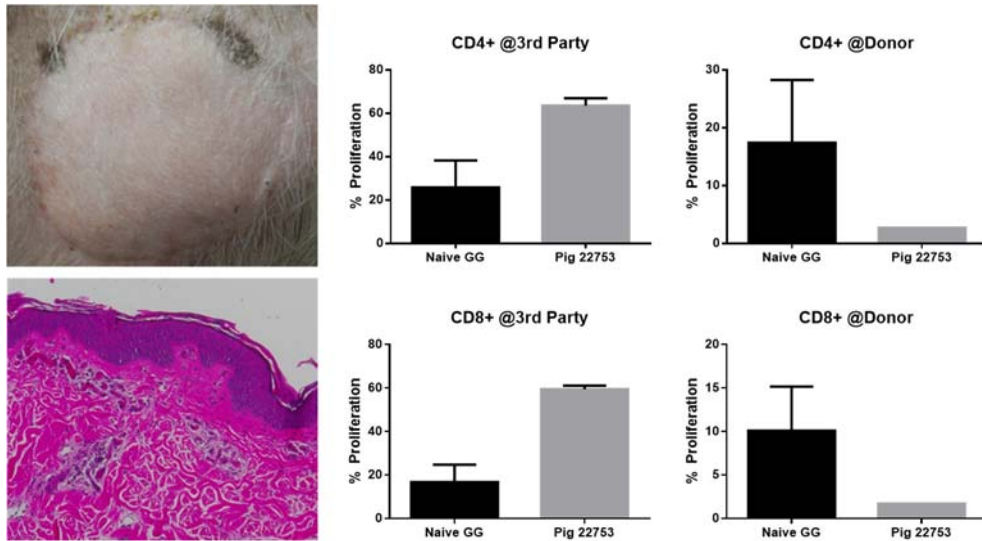


Figure 6: Clinical images of allografts from animals in subtask 2.1: high dose tacrolimus for 60 days followed by withdrawal of immunosuppression. All three animals have been off of tacrolimus since POD 60 and have not demonstrated evidence of rejection.

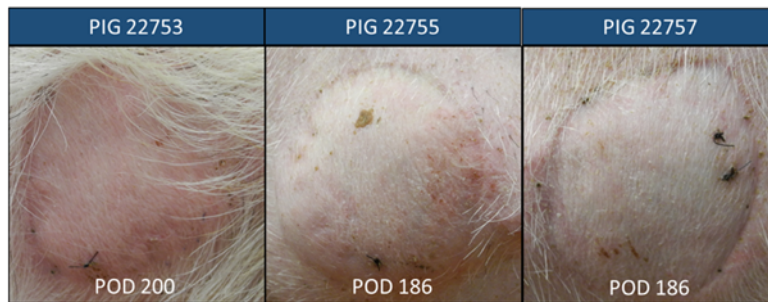
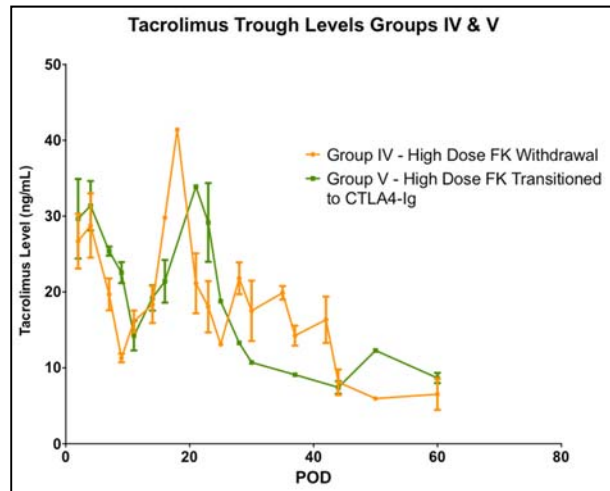


Figure 7: Mean tacrolimus trough levels with standard deviation error bars for animals from subtask 2.1.1 (Group IV, Orange) and 2.2.1 (Group V, Green): high dose tacrolimus (15-20 ng/mL) for 60 days ceased or transitioned to intermittent belatacept, respectively. Note that both groups have similar trends, with high levels for 30 days, which are then gradually weaned and discontinued by POD 60.



2.2 Aim 2, Task 2: Repeat CNI weaning/withdrawal protocol with delayed belatacept treatment and maintenance

All animals in group V (n=5) underwent heterotopic hind limb allotransplantation, were weaned off of CNI on POD60 and transitioned to belatacept maintenance (Figure 3). All animals have completed their course of belatacept maintenance. Representative data from Pig 22843 (Group V) demonstrated evidence of long term graft survival and donor-specific non-responsiveness (Figure 9). The clinical scenario and H&E stain of skin biopsy samples taken on POD204 demonstrated no gross or histologic evidence of graft rejection. MLR of T cell subsets (CD4+ and CD8+) from Pig 22843 PBMC compared to naïve GG pig control was performed, with 3rd party stimulation obtained using PBMC from a DD animal. Both pig 22843 and the naïve GG control animal had robust lymphocyte proliferative response to 3rd party stimulation. However, Pig 22843 had minimal response to PBMC from its donor whereas donor PBMC elicited a strong response in naïve GG animal similar to that with 3rd party stimulation.

Of note, one animal developed infectious polyarthritis and was euthanized according to our ACUC protocol to minimize animal suffering on POD 204. Another animal rejected its graft and was subsequently euthanized on POD 310.

The other three animals were euthanized on POD 360, 347 and 318 with no evidence of graft rejection.

MLRs have been performed on the long term survivors and confirm presence of donor specific unresponsiveness. A representative sample is shown. (Figure 9)

Figure 8: Clinical images of allografts from animals in subtask 2.2: high dose tacrolimus for 60 days followed by transition to belatacept maintenance until POD 150. 3/5 animals have completed the treatment period, and no animal has shown clinical rejection of their grafts. Note that scabbed and erythematous

areas on pigs 22845 are due to trauma to grafts and not rejection (animals have tendency to rub grafts on cages).

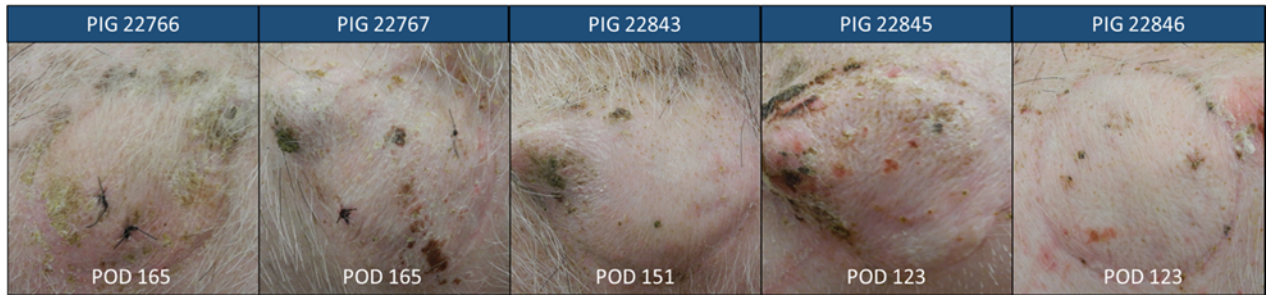
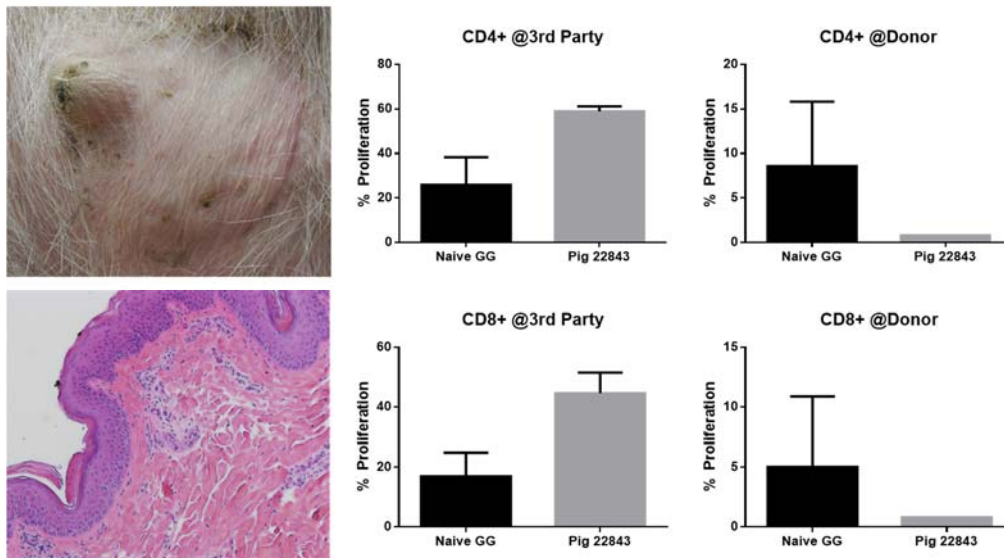


Figure 9: Representative data from Pig 22843 (Group V) demonstrated evidence of long term graft survival and donor-specific non-responsiveness. Clinical image and H&E stain of skin biopsy sample on POD204 demonstrated no gross or histologic evidence of graft rejection. MLR of T cell subsets (CD4+ and CD8+) from pig 22843 PBMC compared to naïve GG pig control. 3rd party stimulation was by PBMC from a DD animal. Both Pig 22843 and naïve GG control animal had robust lymphocyte proliferative response to 3rd party stimulation. Pig 22843 had minimal response to PBMC from its donor whereas donor PBMC elicited a strong response in naïve GG animal similar to that with 3rd party stimulation.



3.1 Aim 3, Task 1: 3.1.1 Perform hind limb transplantation with BMI protocol + short term TAC (30 days) (Group VI; n=6) – in progress – (NCE submitted)

Six animals in Group VI received heterotopic hind limb transplant and were treated as outlined in the SOW Aim 3, Task 1, Subtask 3.1.1.

One animal, Pig 23121 was euthanized on POD0 due to postoperative bleeding and hematoma formation. Another animal Pig 23122 was euthanized on POD4 due to venous thrombosis resulting in graft loss.

One animal 23119 developed post-transplant lymphoproliferative disorder and was euthanized on POD29.

The remaining 3 animals were included in Group VI and received donor bone marrow infusion on POD0 at 60×10^6 cells/kg and short-term high dose tacrolimus (15-20 ng/mL) for 30 days. These animals are now on POD 317, 317 and 373 and have all but one maintained graft survival with no evidence of rejection. One animal, 23124 displayed signs of mechanical trauma approximately around POD 302 (animal was witnessed scratching his graft against the cage), leading to development of erythema, scales and a minor scab. In the subsequent days, the erythema and scaling slowly progressed significantly, involving the whole graft by POD365. We believe that the phenomenon observed was likely due to an unspecific immunological response following the trauma, which triggered skin rejection. A biopsy of the graft was performed on POD365 and results are pending. (Figure 10 and Figure 11). Donor-specific unresponsiveness was confirmed for animal 23223 (Figure 12) and is pending for animal 23224.

Figure 10. Animal and graft survival curves for animals from subtasks 3.1.1 (Group VI: Bone marrow infusion and short term high dose tacrolimus (15-20 ng/mL) for 30 days) and subtask 3.2.1 (Group VIII: Bone marrow infusion and short term high dose tacrolimus for 30 days with perioperative CTLA4Ig on POD0, 2, 4, 6). One animal in Group VI was euthanized on POD29 due to development of PTLD. The 3 remaining animals are now on POD 317, 317 and 373. One animal, 23124 displayed signs of mechanical trauma approximately around POD 302 leading to graft loss by POD365. A biopsy of the graft was performed on POD365 and results are pending. The two Group VIII animals were euthanized on POD 19 and 18, respectively due to respiratory distress, lethargy and failure to thrive.

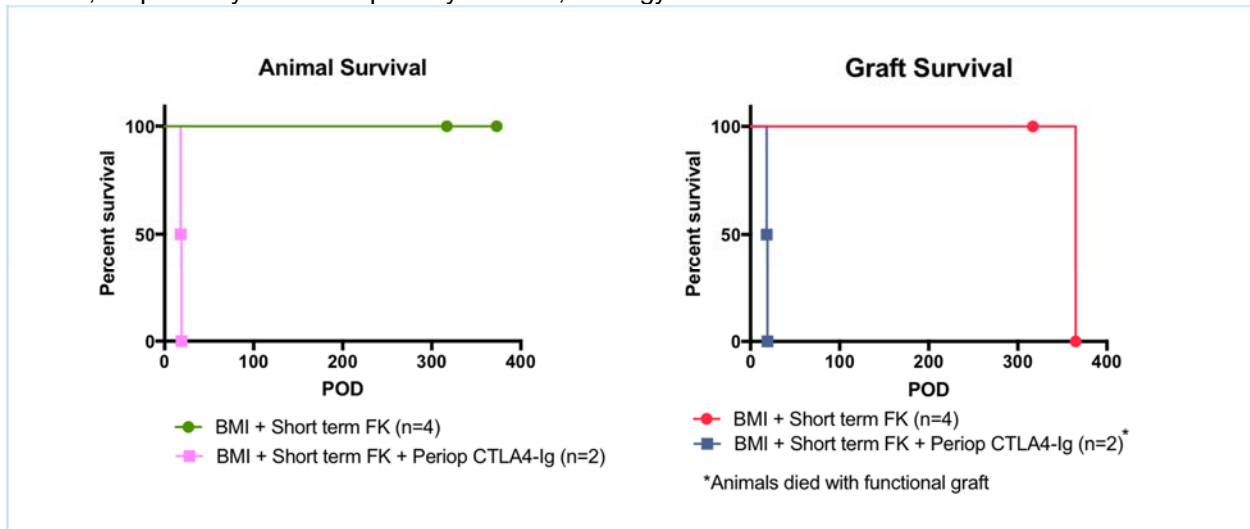


Figure 11: Clinical images of allografts from surviving animals in subtask 3.1.1 BMI and short-term tacrolimus. All 3 animals have been off of tacrolimus since POD30. Currently 2/3 animals appear to have viable grafts with no evidence of rejection. One animal, 23124 showed initial signs of rejection by POD 302 likely initiated by mechanical trauma, which progressed to rejection of the full graft by POD365 (not shown).

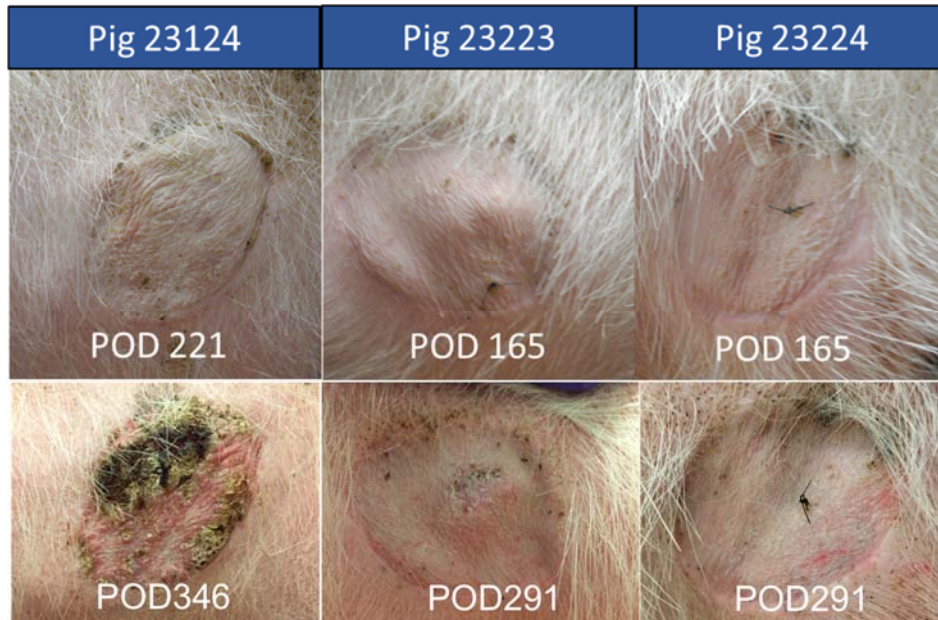
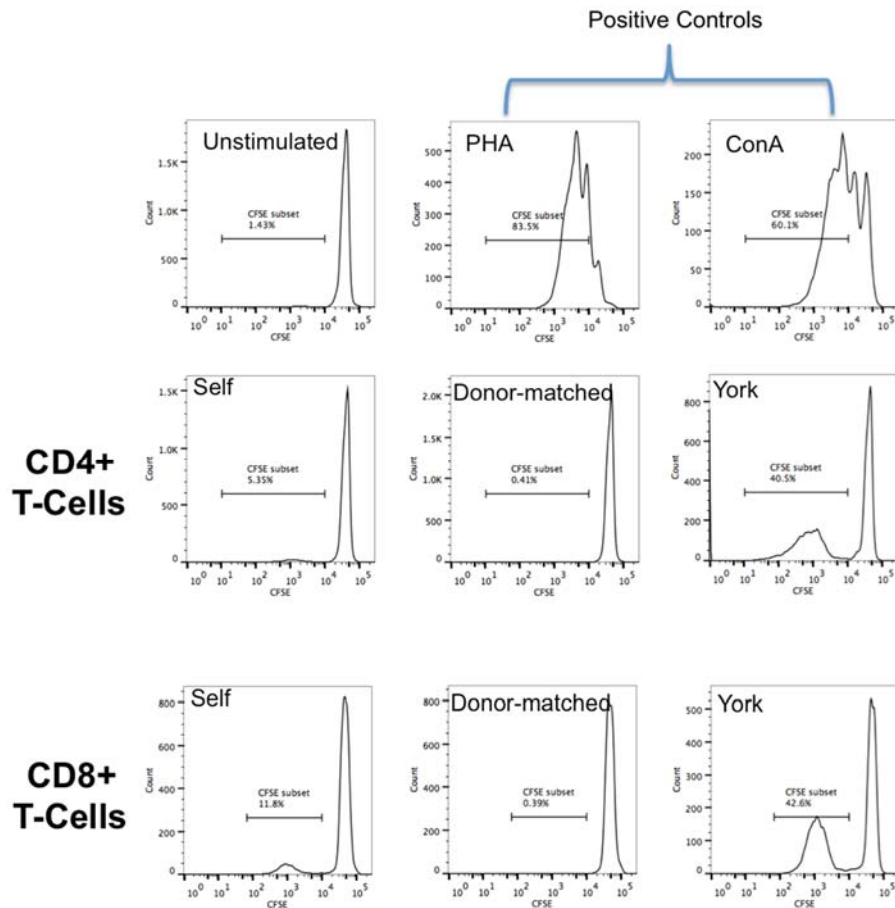


Figure 12: Donor-specific unresponsiveness for animal 23223. CFSE-MLR of the response of responders for 23223 against self, donor-matched PBMCs and third party allogeneic PBMCs from a Yorkshire pig. While response against donor-smatched PBMCs was absent and comparable to the response against self PBMCs, immunocompetence was confirmed by a strong response against Yorkshire PBMCs both at a CD4+ and CD8+T-cell level.



Aim 3, Task 1 – 3.2.1 Perform hind limb transplantation with BMI protocol + short term TAC (30 days) + Perioperative Belatacept (Group VIII; n=2) – (NCE submitted)

Two animals received heterotopic hind limb transplant on 3/4/2016 and were treated as outlined in the SOW Aim 3, Task 2, Subtask 3.2.1.

Both animals completed their course of CTLA4-Ig and were maintained on high-dose tacrolimus after transplant. However, both animals developed lethargy, anorexia, neutropenia and eventual respiratory distress. Despite aggressive supportive therapy in conjunction with our veterinary staff, both animals had to be euthanized due to declining clinical conditions on POD 19 and 18 respectively. Preliminary pathology findings have identified radiation-related cardiac injury as a potential contributor to their clinical outcomes.

c. What opportunities for training and professional development has the project provided?

All co-investigators involved in the project have received training in *in-vivo* and *in-vitro* aspects related to the study. Performing the heterotopic hind limb allotransplants is a complex endeavor requiring expertise in surgical principles, microvascular surgery, and transplant surgery. Preoperative planning and coordination is paramount to success, as well as diligent postoperative care of the animals. Moreover, all co-investigators are gaining knowledge and abilities to manage a complex translational large animal project under supervision of the PI.

Professional development is provided during weekly project updates and laboratory meetings, requiring careful preparation of weekly activities and future plans. Preparation for these presentations to the study group fosters the skill to communicate effectively the details and rationale of the project to other laboratory members. Furthermore, presentations of the results from this study in local and national formats by study group members have further developed young scientist's communication skills.

d. How were the results disseminated to communities of interest?

An abstract detailing the findings detailed above was presented at the International Hand and Composite Tissue Allotransplantation Society (IHCTAS) Annual Meeting on April 16th, 2015 and at the American Transplant Congress in Boston, MA, June 11-15th, 2016.

e. What do you plan to do during the next reporting period to accomplish the goals?

As outlined above, *in-vivo* experiments have been performed according to the statement of work, however, minimal delays in animal availability from the breeder at Massachusetts General Hospital (MGH) have accumulated to contribute to delayed completion of *in-vivo* as well as associated *in-vitro* experiments.

Additionally, unexpected results in survival of animals receiving a VCA combined with short-term immunosuppression, i.e. high-dose tacrolimus, yielded scientifically most promising results. In fact, graft survival significantly exceeded our expectations resulting in long-term/indefinite graft survival in some of the experimental groups. Although to date *in-vivo* experiments have largely been completed, several of these most valuable animals are still ongoing with functioning rejection-free grafts, which we wish to continue monitoring. This encouraging data (outlined in our above in our annual report) highlight the powerful immunomodulatory contributions of grafts containing vascularized bone marrow and indicated that these grafts may be more conducive to immunosuppression sparing protocols as well as tolerance induction.

In light of the aforementioned results and availability of animals, we have submitted a NCE for this project and would like to utilize the additional time to finalize the histology

and immunohistochemistry data to determine graft infiltrating T cell phenotypes, immune phenotyping PBMCs using FACS and analysis of donor specific antibodies. These additional experiments and data analysis will be performed in conjunction with our collaborators at MGH as outlined by the SOW over the next year.

4. IMPACT:

a. What was the impact on the development of the principal discipline(s) of the project?

Our preliminary results are highly encouraging and indicate that CTLA4-Ig has the ability to both allow for CNJ minimization after VCA as well as to maintain rejection free allograft survival after weaning and complete withdrawal of CNJ in patients who have already undergone VCA. In addition, unexpected results have shown that indefinite graft survival can be achieved subsequent to induction and transient high-dose tacrolimus monotherapy in VCAs containing a vascularized bone marrow component or with additional donor bone marrow cell infusion. This could allow to develop alternative protocols devoid of the well known and documented toxicities and side effects of CNJs which are currently hampering broader application of these life changing reconstructive modalities. Furthermore, the inclusion of vascularized bone in our model appears to have an additional immunomodulatory effect as evidenced by the long-term survival of grafts with low dose immunosuppression. Additional clinical follow-up and *in vitro* assays need to be performed to characterize this finding.

b. What was the impact on other disciplines?

Nothing to report

c. What was the impact on technology transfer?

Nothing to report

d. What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

a. Changes in approach and reasons for change

Nothing to report

b. Actual or anticipated problems or delays and actions or plans to resolve them

Please see outline of future experiments for next report period above.

c. Changes that had a significant impact on expenditures

Our rate of expenditure has increased from the previous annual report as we have performed a significant number of transplants along with the postoperative treatment regimen (primarily belatacept). Furthermore, *in-vitro* analysis is fully underway and the cost of reagents will be expected to increase in the upcoming year. In addition, due to the encouraging long-term survival outcomes housing costs have been higher than initially anticipated.

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

e. Significant changes in use or care of human subjects

Nothing to report

f. Significant changes in use or care of vertebrate animals.

Nothing to report

g. Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?

Name: Gerald Brandacher

Project Role: Principal Investigator

Nearest Person Month Worked: 10%

Contribution to Project: Dr. Brandacher oversees all aspects of project planning, execution and data analysis. He actively participated in all animal surgeries.

Funding Support: Grant

Name: W. P. Andrew Lee

Project Role: Co-Investigator

Nearest Person Month Worked: 2%

Contribution to Project: Dr. Lee participated in project planning and data analysis.
Funding Support: Departmental Sources

Name: Justin Sacks
Project Role: Co-Investigator
Nearest Person Month Worked: 5%
Contribution to Project: Dr. Sacks participated in all donor surgeries.
Funding Support: Departmental Sources

Name: Jaimie Shores
Project Role: Co-Investigator
Nearest Person Month Worked: 5%
Contribution to Project: Dr. Shores participated in all recipient surgeries.
Funding Support: Departmental Sources

Name: Damon Cooney
Project Role: Co-Investigator
Nearest Person Month Worked: 5%
Contribution to Project: Dr. Cooney participated in all recipient surgeries, post-transplant care and data analysis.
Funding Support: Departmental Sources

Name: Howard Wang
Project Role: Post-Doctoral Fellow
Nearest Person Month Worked: 50%
Contribution to Project: Dr. Wang participated in all recipient surgeries, pre and post-transplant care, performed *in vitro* assays and data analysis.
Funding Support: Grant

Name: Angelo A. Leto Barone
Project Role: Post-Doctoral Fellow
Nearest Person Month Worked: 50%
Contribution to Project: Dr. Leto Barone participated in post-transplant care, performed *in vitro* assays and data analysis.
Funding Support: Grant

Name: BC Oh
Project Role: Faculty
Nearest Person Month Worked: 25%
Contribution to Project: Dr. Oh participated in all recipient surgeries, pre and post-transplant care, performed *in vitro* assays and data analysis.
Funding Support: Grant

b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

c. Partnering Organization

i. Organization Name: Massachusetts General Hospital

ii. Location of Organization: Boston, MA

Massachusetts General Hospital provided support and consultation with regard to donor/recipient selection and matching as well as post-transplant immunological *in vitro* assays.

8. SPECIAL REPORTING REQUIREMENTS

a. **QUAD CHARTS:** Attached.

9. APPENDICES

Nothing to report.

Immunomodulation and Tolerance Induction after VCA Using Biologic Agents (CTLA4-Ig) and Donor Bone Marrow Cells

MR120034P10, Restorative Transplantation Research

Award Number: W81XWH-13-2-0060

PI: Gerald Brandacher, M.D.

Org: Johns Hopkins University School of Medicine Award Amount: \$1,297,034



Study/Product Aim(s)

- Establish a belatacept-based protocol to enable CNI minimization after Vascularized Composite Allograft Transplantation (VCA).
- Investigate the possibility to convert from conventional CNI-based immunosuppression to belatacept maintenance with subsequent CNI withdrawal.
- Compare immunomodulatory donor bone marrow (BM) infusion (BMI) to BM transplantation (BMT) with establishment of durable mixed chimerism for induction of tolerance and/or VCA survival on CNI-free immunosuppression using a belatacept-based regimen.

Approach

In this study we propose to develop novel protocols using donor bone marrow cells and FDA-approved biologic agents (Cytotoxic T-lymphocyte-associated antigen-4 immunoglobulin [CTLA4-Ig], belatacept) for the induction of immune tolerance with minimal or only transient immunosuppression in de-novo VCA recipients and to allow withdrawal of calcineurin inhibitors (CNIs) from patients that have already been transplanted under conventional immunosuppression. Studies will be performed using a translational large animal model for VCA as outlined in Figure 1.

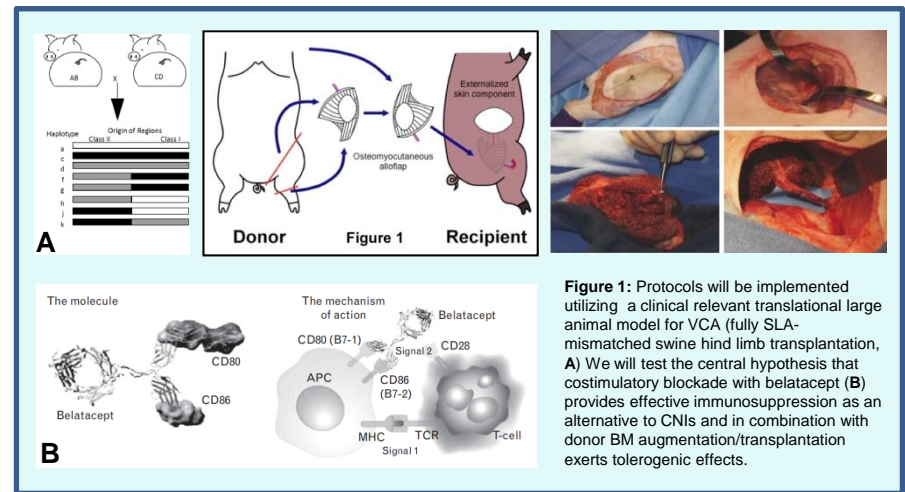


Figure 1: Protocols will be implemented utilizing a clinical relevant translational large animal model for VCA (fully SLA-mismatched swine hind limb transplantation, **A**) We will test the central hypothesis that costimulatory blockade with belatacept (**B**) provides effective immunosuppression as an alternative to CNIs and in combination with donor BM augmentation/transplantation exerts tolerogenic effects.

Accomplishment: Implemented successfully first clinical protocol for upper extremity transplantation using donor bone marrow cell therapies and tacrolimus monotherapy.

Timeline and Cost

Activities	CY	14	15	16
Establish a belatacept-based protocol to enable CNI minimization after VCA (JHU)				
Test ability to convert from conventional CNI-based immunosuppression to belatacept maintenance with subsequent CNI withdrawal (JHU)				
Use BMI combined with CNI-free belatacept-based regimen to establish chimerism and tolerance induction after VCA (JHU)				
Use BMT combined with CNI-free belatacept-based regimen to establish chimerism and tolerance induction after VCA (MGH)				
Estimated Budget (\$K)		\$379,907	\$366,875	\$633,096

Updated: (10/15/2016)

Goals/Milestones

CY14 Goals – CNI minimization

- ✓ Adapt clinically established induction and CNI maintenance regimen in this translational large animal VCA model
- ✓ Determine impact of peri-transplant belatacept treatment to allow for allograft survival with low-dose CNI treatment

CY15 Goals – CNI withdrawal

- ✓ Attempt CNI weaning/withdrawal without CTLA4-Ig and assess time course of allograft rejection
- ✓ Perform CNI weaning/withdrawal with delayed belatacept treatment and maintenance

CY16 Goals – CNI free immunosuppression and tolerance induction

- Determine impact of BMI vs. BMT combined with short-term CNI on immunomodulation and allograft survival
- Develop tolerance protocol combining optimized BM regimen with short-course CNI and peri-transplant belatacept treatment
- Develop tolerance protocol combining optimized BM regimen with short-term CNI, peri-transplant and short course post-transplant belatacept treatment

Comments/Challenges/Issues/Concerns

- Experiments as outlined by SOW are in progress, validation and adaptation of induction regimen completed

Budget Expenditure to Date

Projected Expenditure: NA

Actual Expenditure: \$1,170,391