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TITLE: Autologous Hematopoietic Stem Cell Transplantation to Prevent Antibody-Mediated Rejection after Vascularized Composite Allotransplantation

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14. ABSTRACT

The investigators successfully performed initial control experiments in Group 1, unsensitized animals applying the proposed induction and maintenance immunosuppressive regimen to assess its safety and feasibility for maintaining VCA allograft survival in this model. Successful VCA (i.e. swine heterotopic hind limb) transplantation in Group 2 were performed across a full SLA mismatch in the setting of donor skin sensitization. The animals were treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml for 30 days.

15. SUBJECT TERMS

vascularized composite allotransplantation, sensitization, autologous hematopoietic stem cell transplantation, antibody mediated rejection, donor specific antibodies

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PI: Gerald Brandacher, MD

1. INTRODUCTION

For many devastating combat injuries where conventional reconstruction is not possible, Vascularized Composite Allotransplantation (VCA) has become a viable alternative. This approach provides new, exciting options for Wounded Warriors that could restore appearance, anatomy, and function better than other available treatment options. However, clinical management of these injuries prior to transplantation frequently requires multiple blood transfusion or skin grafts resulting in the formation of alloantibodies (anti-HLA IgG Abs) and sensitization. In solid organ transplantation (SOT), such pre-sensitization is the greatest risk factor for allograft rejection and long-term graft failure, and causes patients to be excluded as candidates for transplantation. However, the role of donor-specific antibodies (DSA) and mechanisms of antibody-mediated rejection (AMR) in VCA are largely unknown. Thus, there is an imminent need to develop a better understanding of the mechanisms related to DSA and AMR after VCA as well as to implement novel clinically relevant desensitization protocols that would be applicable to a cadaveric donor setting.

The objective of this project therefore is to comprehensively investigate the mechanisms and impact of pre-existing and de-novo DSA and AMR in VCA and to develop a clinically relevant desensitization protocol that will subsequently broaden the population of sensitized patients eligible for reconstructive transplantation. The investigators will test their central hypothesis that that the impact and mechanisms, of AMR in reconstructive transplantation as well as the cadaveric donor setting will require specifically tailored desensitization strategies and treatment regimens in order to improve access and outcomes for highly sensitized VCA candidates in a pre-clinical large animal model.

2. KEYWORDS

vascularized composite allotransplantation, sensitization, autologous hematopoietic stem cell transplantation, antibody mediated rejection, donor specific antibodies

3. ACCOMPLISHMENTS

During year two of this project a total of four successful VCA (i.e. swine heterotopic hind limb) transplantations were performed across fully SLA mismatched donor and recipient pairs. The recipients were sensitized with SLA disparate donor skin grafts to achieve donor-specific presensitization. Heterotopic hind limb transplantations were subsequently performed 25 days post skin transplantation. Recipient animals were treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml until rejection. Additionally, we have performed 2 more swine heterotopic hind limb transplantation for Group 1.

The experienced delays were required us to seek a No Cost Extension (NCE) plus re-scoping the current SOW for this project which we have submitted. We have been in contact with the program officer to discuss the timeline and required documents for this process.

A. Major Goals

The major goals of this project for Year 2 as stated in the approved SOW were:

Major Task 1: Identify the role of presensitization, DSA, and mechanisms of AMR in VCA
Major Task 2: Identify the role of de-novo DSA and impact on AMR in VCA

Major Task 3: Implement a novel clinically relevant desensitization protocol using autologous hematopoietic stem cell transplantation (HSCT)

Table 1: Progress against the SOW

Task	**Start Date**	**End Date**	**% Complete**	**Comments**
Major Task 1	October 2017	October 2018	100 %	Major task 1 has been completed as detailed in the previous quarterly reports with three recipients successfully performed in Group 1 and two recipients in Group 2, respectively. Although there has been a total of five recipients proposed in Group 2, the clinical outcome in the first two animals was consistent and as expected with rapid graft loss in the sensitized animals. We therefore propose to not perform any additional control animals in this group as outlined in our submitted NCE and change in scope of work request.
Major Task 2	-	-		Given the significant increase in price per animal we will have to reduce the overall number of animals/transplants proposed in the original SOW. In order to be able to not compromise statistical power

				in the individual experimental groups we therefore propose to forgo Major task 2 and to focus on completion of the clinically more relevant Major task 3 for the remainder of the project.
Major Task 3	September 2018	October 2019	0 %	Major task 3 will be performed as originally proposed with a total of six transplants performed in Group 5. However, according to our preliminary study on harvesting autologous bone marrow in Group 5 to obtain sufficient bone marrow cell numbers for autologous BM transplantation, we will require three additional SLA-matched bone marrow donor animals at a ratio of 1 donor per 2 transplant recipients.

B. Accomplishment of Goals

Subtask 1.2 - Perform swine heterotopic hind limb transplantation (Groups 1, 2); monitor kinetics of circulating DSA (flow cytometry) in sensitized recipients after VCA, and characterize DSA (IgG) and C3d/C4d deposition (immunofluorescence) in tissue components of VCA

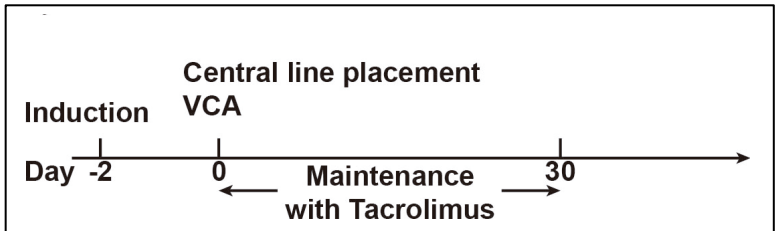

Figure 1: Treatment regimen of the recipient animal

Successful VCA (i.e. swine heterotopic hind limb) transplantation was performed across a full SLA mismatch combination (Donor: SLA LL, Recipient: SLA CC). The animal assigned to Group1 was treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml for 30 days (**Figure 1**). The placement of a central venous access catheter allowed for sequential blood sampling for further assessment of tacrolimus trough levels and collection of serum and PBMCs (peripheral blood mononuclear cells) at defined time points (POD -1, POD 0, 1, 3, 5, 7,

10). Grafts were monitored by daily clinical assessment and photo documentation to determine any macroscopic changes to the allograft. Skin biopsy was performed at POD0, 7. As shown in **Figure 2**, the allograft was maintained without any evidence of rejection until POD13 under tacrolimus treatment.

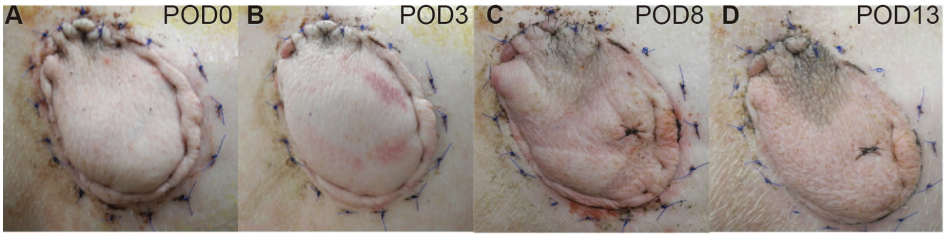

Figure 2: Clinical photos of skin component of VCA allograft

Tacrolimus blood levels targeting 10-15ng/ml was well maintained without clinical evidence of rejection or any clinical side effects as shown in **Figure 3**. To confirm allo-responsiveness between donor and recipient, we performed mixed lymphocyte reaction (MLR) prior to transplantation using PBMCs. As shown in **Figure 4**, PBMCs from the recipient responded well against donor stimulation as well as against a third-party (York strain) control.

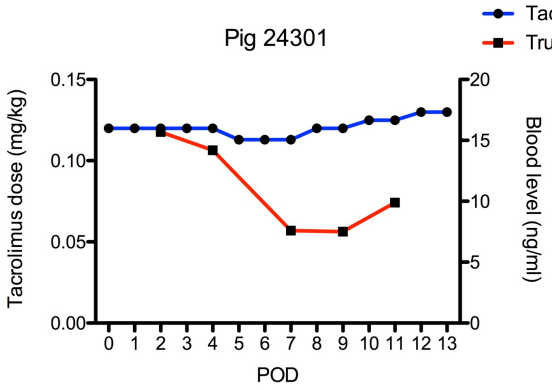

Figure 3: Tacrolimus dose and trough blood levels

Although the recipient animal was doing well without any clinical signs of side effects or toxicities from the tacrolimus treatment and without any evidence of graft rejection until POD13 the animal was found dead the next morning. A necropsy was performed but unfortunately was not able to reveal the cause of death or any significant pathology especially no obvious signs of infection.

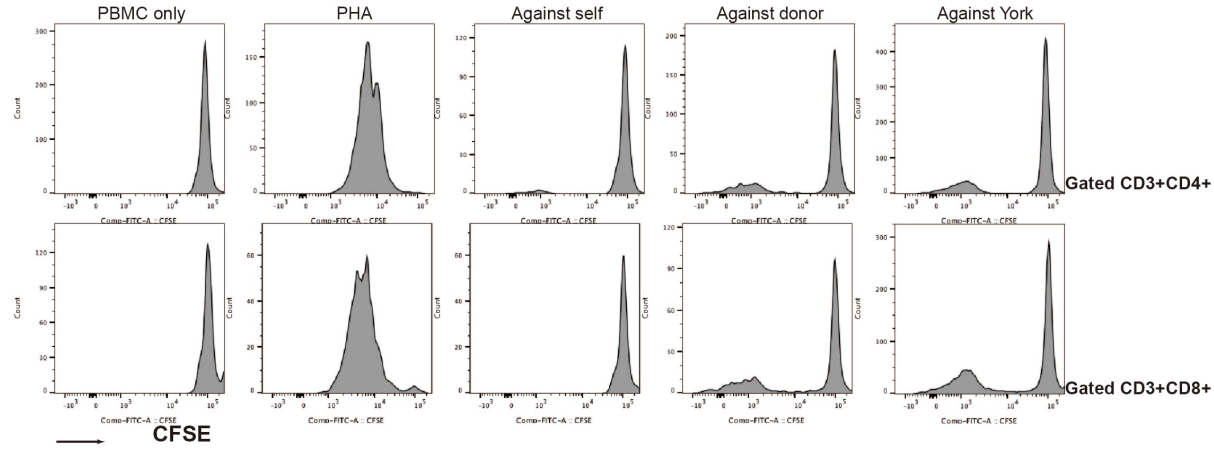

Figure 4: Mixed lymphocyte reaction (MLR) showing donor hypo-responsiveness

Another swine heterotopic hind limb transplantation (Donor: SLA CC, Recipient: SLA AD) was performed for Group 1. Induction therapy including a total of 50 cGy of total body irradiation and 350 cGy of thymic irradiation was applied at POD -1 in an effort to decrease the pre-existing alloreactive cells. As shown in **Figure 5**, tacrolimus blood levels targeting 10-15ng/ml were well maintained without clinical evidence of rejection or any clinical side effects.

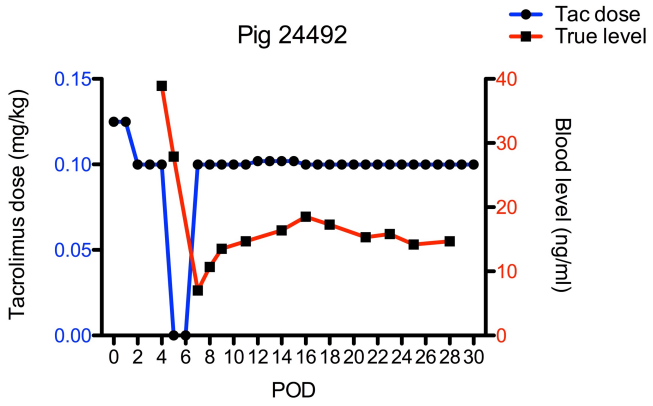

Figure 5: Tacrolimus dose and trough blood levels

As shown in **Figure 6**, the VCA allograft is maintaining without rejection over POD50 after 30 days of Tacrolimus treatment.

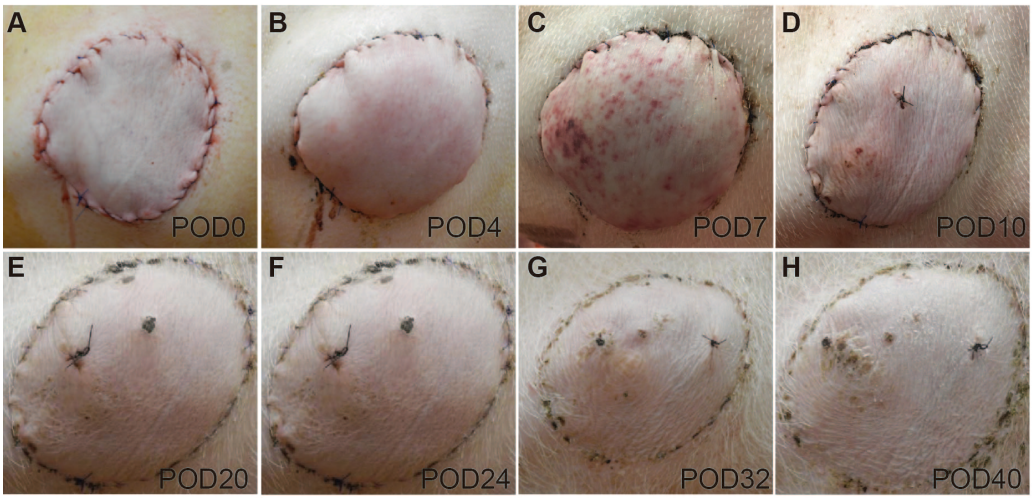

Figure 6: Clinical photos of skin component of VCA graft

Furthermore, to determine alloreactivity between donor and recipient we performed *in-vitro* MLR assays (**Figure 7**) prior to transplantation indicating a viable donor-specific proliferative response of recipient T cells as well as post transplantation indicating donor-specific quiescence of recipient CD4 and CD8 T cells.

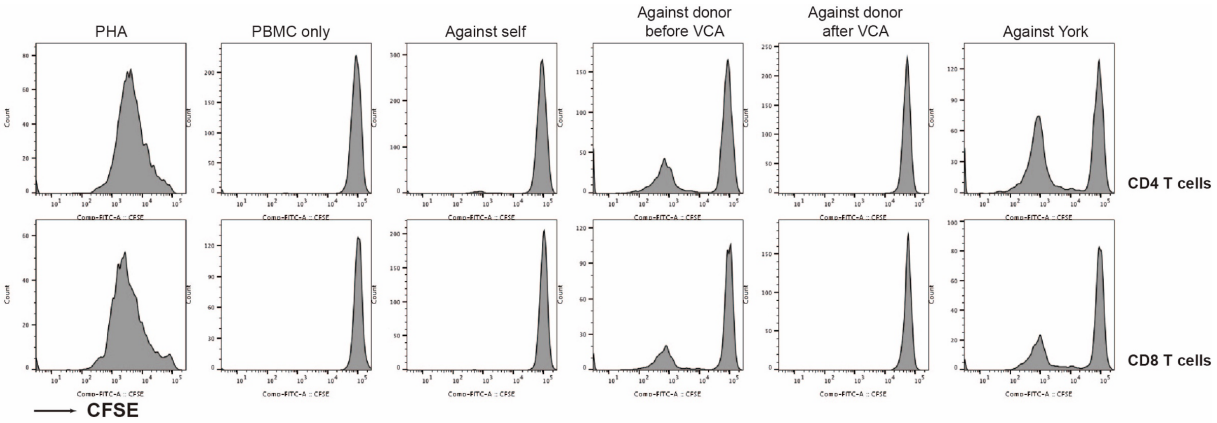

Figure 7: Mixed lymphocyte reaction (MLR) showing donor hypo-responsiveness

Subtask 1.3- Evaluate histopathological changes (H&E) and inflammatory infiltration (IHC or IF for CD3, CD4 and CD8 (T cells), ED1 (circulating macrophages), ED2 (resident macrophages), CD19 (B cells) in pre-sensitized VCA recipients

Skin biopsies from animal 24492 in Group 1 were taken on POD 0, 7, 14, 30 and fixed in 10% PFA and embedded in paraffin for downstream analysis (**Figure 8**). **Figure 9** shows examples of immunohistochemistry stainings to assess CD3 T cells, CD20 B cells, and Foxp3 regulatory T cell infiltration in the skin component obtained with the protocols that were established by the investigators. In preparation of upcoming experiments, we have continued to optimize immunohistochemistry staining.

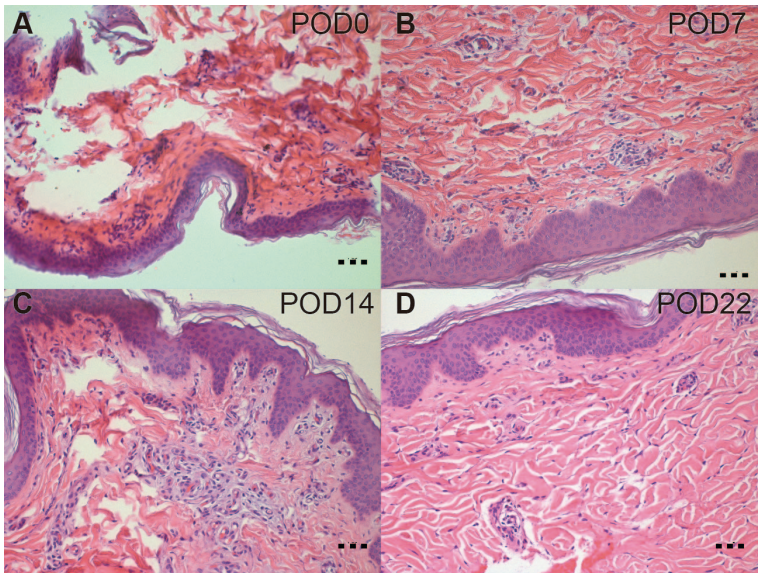

Figure 8: Histological photos of skin biopsy in VCA graft

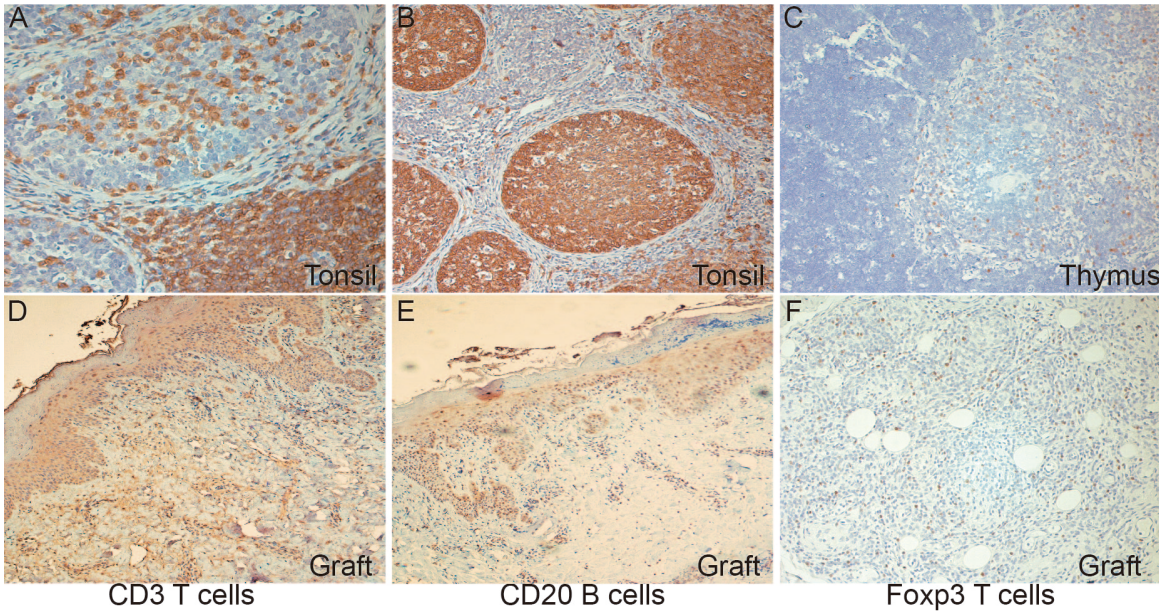

Figure 9: Immunohistochemistry staining for CD3 T cells, CD20 B clls and Foxp3 regulatory T cells in the skin component of heterotopic hind limb transplant recipients.

Subtask 2.1- Perform swine heterotopic hind limb transplantation (Group 3), monitor kinetics of circulating DSA (flow cytometry) in sensitized recipients after VCA, and characterize DSA (rat IgG) and intragraft C3d/C4d deposition (immunofluorescence)

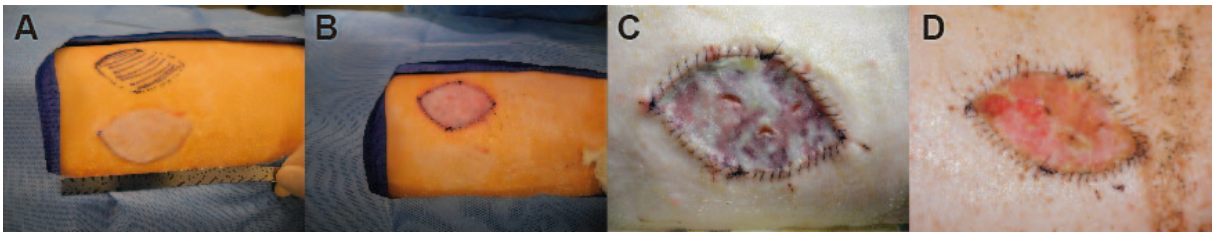

Figure 10: Clinical photos of skin area after skin sensitization. **A**. Example of skin sensitization, **B**. After skin sensitizatin, **C**. 7 days after skin sensitization, **D**. 14 days after skin sensitization

Under Major Task 2, Subtask 2.1, we performed two heterotopic swine hind limb transplantations during this reporting period across a full SLA mismatch combination (Donor: SLA CC, Recipient: SLA AD) and enrolled the animals into Group 3 as sensitized controls with standard immunosuppressive treatment. Treatment for the animal consisted of non-myelobablative total body irradiation (50cGy) and thymic irradiation (350 cGy) two days prior to transplantation as well as continuous immunosuppressive therapy using tacrolimus for 30 days. In this current set of *in-vivo* experiments, skin transplantation from SLA disparate animals was performed to achieve donor-specific presensitization. Simultaneously with skin transplantation a central venous catheter was placed to allow for sampling of whole blood to obtain peripheral blood mononuclear cells (PBMCs) and serum. Animals were monitored clinically and photo documentation of the skin allograft was performed (**Figure 10**). The formation and kinetics of donor specific antibodies (DSA) was monitored after skin transplantation using flow cytometry-based antibody cross match analysis. Significant levels of donor specific IgM and IgG antibodies were detected proving successful donor specific allosensitization of the recipient animals (**Figure 11**). Thus, 25 days post skin transplantation, heterotopic hind limb transplantation was performed into both pre-sensitized recipients using the hind limbs of the original skin graft donor (SLA CC to SLA AD). The animals were treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based

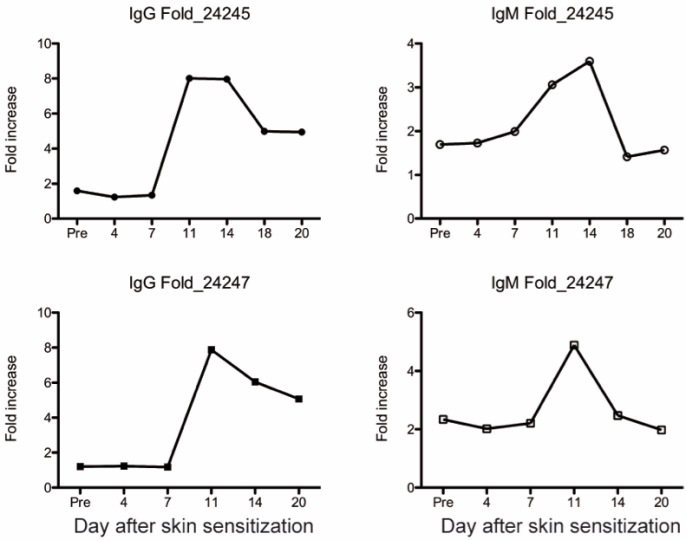

Figure 11: *In vitro* monitoring of serum DSA (IgG and IgM) formation after skin sensitization

immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml until rejection (**Figure 12**).

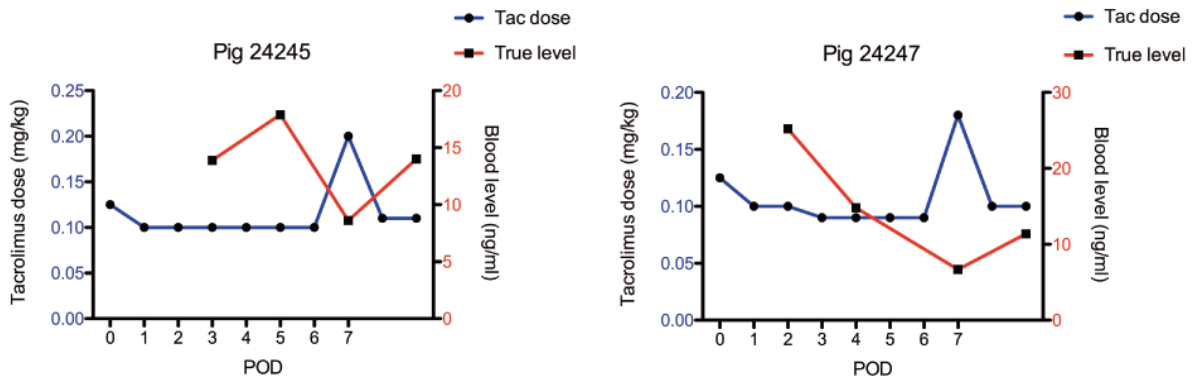

Figure 12: Clinical monitoring of tacrolimus blood level

Clinical observation of the VCA allograft survival demonstrated accelerated graft rejection with >90% skin necrosis after 9-10 days (**Figure 13**), despite therapeutic tacrolimus-based immunosuppression with target trough levels of 10-20 ng/ml as outlined by **Figure 12**. Serum and PBMCs were harvested and stored for further downstream analysis of cellular and humoral markers of allograft rejection and maintenance. To determine cellular alloreactivity amongst donor and recipient, we performed *in-vitro* MLRs demonstrating pre-transplant donor specific responsiveness which was maintained after sensitization by skin transplantation as well as after VCA (**Figure 14**). Rebounding donor specific IgG and IgM formation was demonstrated after VCA by repeat *in-vitro* flow cross-match (**Figure 15**). Skin biopsies were taken on POD 0, 7, and day of rejection and fixed in 10% PFA and embedded in paraffin for downstream immunohistochemistry analysis. Initial H&E stained sections are shown in **Figure 16**.

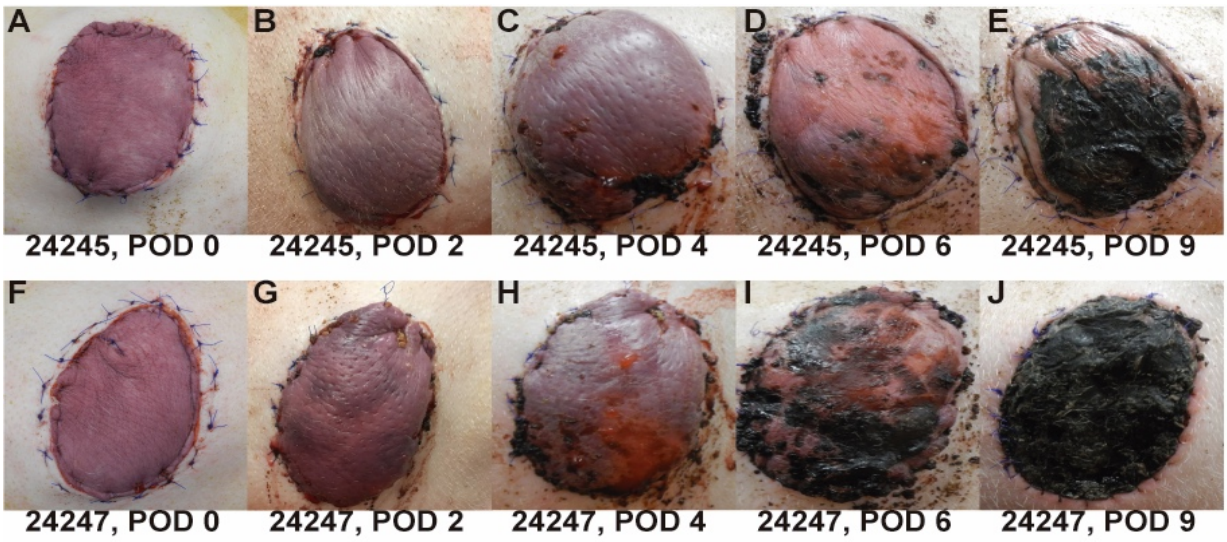

Figure 13: Clinical photos of skin component of rejecting VCA allograft

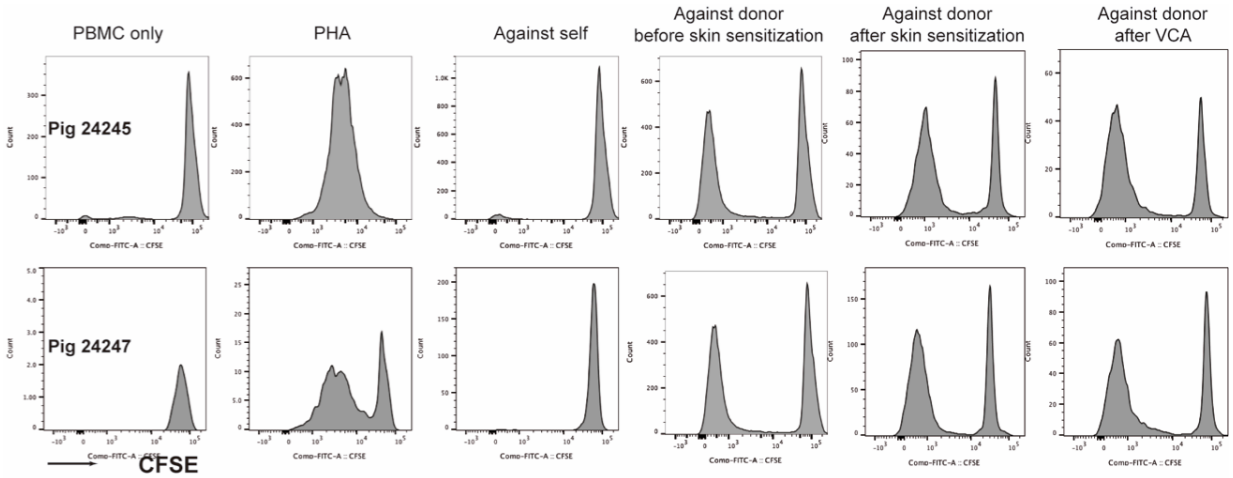

Figure 14: Mixed lymphocyte reaction (MLR) showing donor specific memory responsiveness

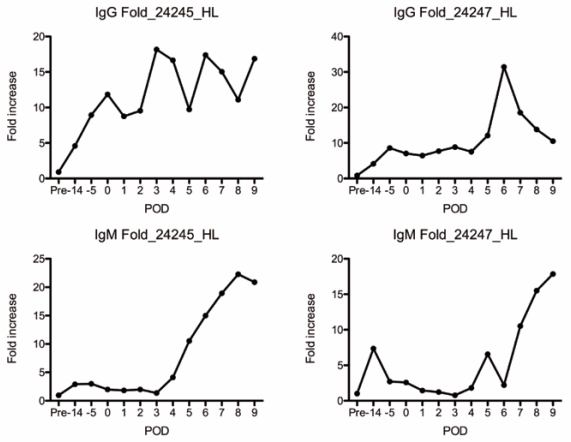

Figure 15: *In vitro* monitoring of serum DSA (IgG and IgM) formation after VCA in presensitized recipients

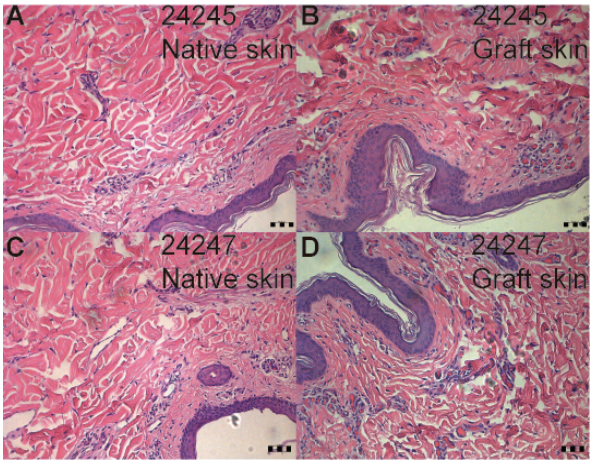

Figure 16: Histological photos of skin biopsies in VCA.

As shown in **Figure 17**, kidney and skin biopsy samples from skin-presensitized recipient showed C4d deposition in the area of vessel endothelium. Further optimization in various immunohistochemistry staining is ongoing.

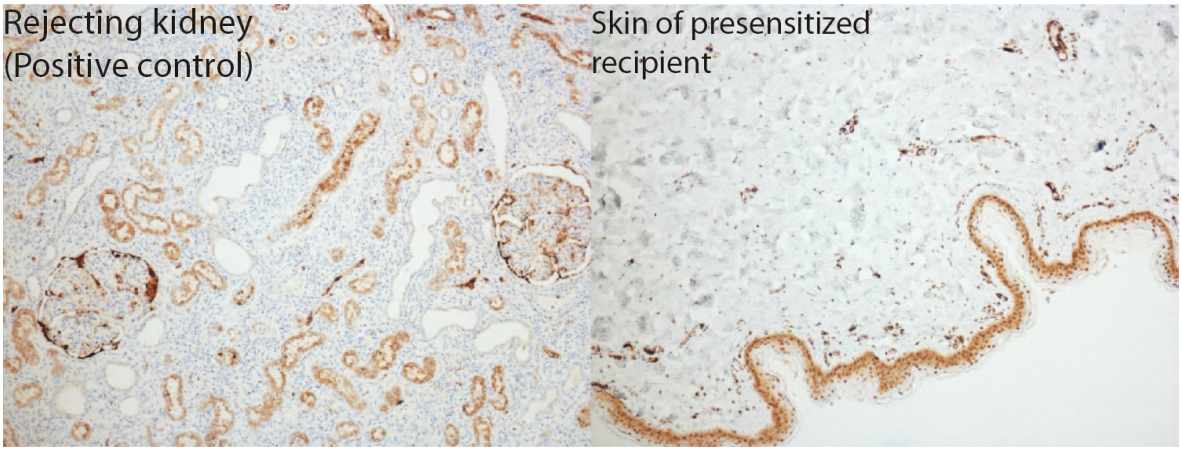

Figure 17: Immunohistochemistry staining for C4d deposition in the tissues.

Significant results or key outcomes

The investigators successfully performed initial control experiments in unsensitized animals applying the proposed induction and maintenance immunosuppressive regimen to assess its safety and feasibility for maintaining VCA allograft survival in this model. Moreover, the investigators performed two heterotopic swine hind limb transplantations during this reporting period across a full SLA mismatch combination and enrolled the animals into Group 3 as sensitized controls with standard immunosuppressive treatment proving accelerated memory response due to donor specific pre-sensitization.

Unfortunately, due to the lack of available animals from the breeder at MGH/Columbia no further transplants could be performed (see detailed explanation below). Given the lack of available animals for transplant the investigators optimized *in vitro* assays and methodologies such as mixed lymphocyte reaction, immunohistochemistry staining for T, B and regulatory cells, detection of donor specific antibodies.

Other achievements

C. Training and Professional Development

The completion of Task 1 of the SOW has provided the PI's with the opportunity to solidify the training of the involved research fellows with regards to optimizing both microsurgical technique (swine heterotopic hind limb transplantation model) as well as advanced *in-vitro* assays for the assessment of donor-specific response.

D. Result Dissemination

Nothing to report

E. Future plan

Since the costs of swine from the breeder at Columbia have significantly increased as compared to what initially has been budgeted when the breeder was still at MHG and according to our preliminary study on harvesting autologous bone marrow in Group 5 to obtain sufficient bone marrow cell numbers for transplantation, the investigators proposed to re-scope the current SOW together with a no-cost extension request for one year.

4. IMPACT

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

The development of specifically designed animal models as proposed in this study will be a prerequisite to pave the way to the acquisition of the lacking DSA and AMR data indispensable to the further advancement of field of VCA. The insights gained from this project will lead to a better understanding of the molecular and pathological sequelae of DSA and AMR in VCA. This will bring us closer to developing specific, targeted, and clinically applicable treatment modalities for AMR. In particular, the use of autologous HSCT as a novel, rapidly translatable desensitization approach will have a significant impact on our discipline and will allow us to successfully perform VCA in highly sensitized patients who otherwise would be excluded as candidates for transplantation.

B. Impact on Other Disciplines

A better understanding of DSA and AMR in VCA, along with the development of clinically applicable desensitization 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 to enable desensitization in a cadaveric donor setting.

C. Impact on Technology Transfer

Nothing to Report

D. Impact on Society beyond Science and Technology

Nothing to Report

5. CHANGES/PROBLEMS

Nothing to Report

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

As described in previous Quarterly Reports, we have been experiencing significant delays with Major Tasks 1 and 2 due to limited animal availability from the breeder at Columbia University. Specifically, the MGH Miniature Swine herd, previously owned and managed by Massachusetts General Hospital (MGH) and Dr. David Sachs was transferred to New Jersey and ownership was transferred to Columbia University, New York. This change in proprietorship has led to significant delays in availability of suitable animals for transplantation due to low breeding activity which has greatly affected our ability to perform the large animal experiments as proposed by the SOW in a timely fashion. This situation has most recently been aggravated due to the fact that an infectious disease outbreak within the breeding herd has been reported from Columbia University. Although currently the viral symptoms have been largely cleared available animals are still scarce. Specific breeding efforts have been initiated but thus far have been not sufficient to meet our demands for this study. We will continue to work with the breeder to advance those initiatives to assure continued access to the animals. Based on several phone conversations with Mr. Scott Arn and the staff from the breeding facility it is expected that animals will be available in the next couple of months.

To carefully address the specific needs of the experiments (SLA type, PAA+, gender, size, age) as outlined by the SOW specific breeding pairs to allow for reproducibility are required and the investigators have been assured that those will be provided for this project. As our team has extensive experience with the proposed large animal experiments we don't foresee any additional issues in order to accelerate the pace of the proposed transplants once animals become available from Columbia.

In addition, to this limited availability of suitable large animals for transplantation, the change in administrative oversight led to alterations in the price structure of the new breeder at Columbia University resulting in a substantial and unanticipated cost increases from $1,600 to $2,500 per animal. As a result, the investigators propose to adjust and change the scope of work (SOW) which has been approved on 09/25/2018.

C. Changes that had a Significant Impact on Expenditures

Nothing to Report

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

Nothing to Report

6. PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

A. Participants & Collaborators

Name (First and Last)	Description
Gerald Brandacher, M.D.	No Change
Zhaoli Sun, M.D.	No Change
Byoungchol Oh, D.V.M. Phd	No Change
Giorgio Raimondi, Ph.D.	Project Role: Co-Inv Nearest person month worked: 1 Contribution to Project: Dr. Raimondi participated in data collection, data interpretation, and supervision of the post doc fellow involved in this project.
Damon Cooney, M.D., Ph.D.	Project Role: Co-Inv Nearest person month worked: 1 Contribution to Project: Dr. Cooney participates in the large animal surgeries, data interpretation, as well as preparation and maintenance of the animal protocol.

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

Name (First and Last)	Current Support Changes
Gerald Brandacher	Change: Received – Deceased Donor Bone Marrow Salvage and Processing Subgrant: Ossium Health / Prime: NIAID Award : 1R43AI129444-01A1 Role: PI / Effort: 5% Date: 01/01/18 – 12/31/18
Gerald Brandacher	Change: Received – Long Term Banking of Vascularized Composite Grafts using ice-free cryopreservation by vitrification and nano-warming tehcnologies Subgrant: Tissue Testing Technologies / Prime: USAMRMC Award: W81XWH-16-1-0508 Role: PI / Effort: 1% Date: 01/01/18 – 09/30/18
Gerald Brandacher	Change: Received – Optimal rewarming solution for cryopreserved tissue systems Subgrant: Tissue Testing Technologies / Prime: DoD Award: W81XWH-16-C-0074 Role: Co- Investigator / Effort: 5%

	Date: 04/01/18 – 09/14/18
Gerald Brandacher	Change: Received – Phase II: Non-Toxic, Highly Effective Bioinspiried Cryoprotectants Subgrant: X Therma / Prime: USAMRMC Award: W81XWH-16-C-0066 Role: PI / Effort: 5% Date: 08/01/18 – 01/31/19
Giorgio Raimondi	Change: Received – Phase II: Non-Toxic, Highly Effective Bioinspiried Cryoprotectants Subgrant: X Therma / Prime: USAMRMC Award: W81XWH-16-C-0066 Role: PI / Effort: 2% Date: 08/01/18 – 01/31/19
Gerald Brandacher	Change: Received – Novel Cell Therapy-based Strategies to Induce Immune Tolerance in VCA Award: Kaohsuing Medical University Hospital Role: PI / Effort: 9% Date: 07/01/18 – 06/30/19

C. Other organizations involved as partners

Nothing to Report