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PRINCIPAL INVESTIGATOR: Gerald Brandacher, MD

CONTRACTING ORGANIZATION: Johns Hopkins University
BALTIMORE, MD 21218

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Gerald Brandacher, M.D.

E-Mail:gbranda2@jhmi.edu

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14. ABSTRACT

Close to 40% of combat injuries sustained in Operation Iraqi Freedom and Operation Enduring Freedom involve severe extremity and craniofacial trauma. For many devastating injuries where conventional reconstruction is not possible, vascularized composite allotransplantation (VCA) has become a viable alternative, providing new, exciting options for Wounded Warriors that could better restore the appearance, anatomy, and function. However, clinical management of these injuries prior to reconstruction frequently requires multiple blood transfusion or skin allografts resulting in the formation of alloantibodies (anti-HLA IgG Abs, donor specific antibodies or DSA) and a high degree of sensitization. The role of DSA and mechanisms of antibody mediated rejection (AMR) in VCA are still largely unknown. To date, there is only one single experimental study published that has recently attempted to define the role of DSA in a rat model of vascularized osteomyocutaneous flap allotransplantation. As such, this project aims to comprehensively investigate the mechanisms and impact of pre-existing and de-novo DSA and AMR in in VCA. The goal is to develop a clinically translatable desensitization protocol that will subsequently broaden the eligible population for reconstructive transplantation to include those patients who have become sensitized to foreign antibodies

15. SUBJECT TERMS

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

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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 the last year of this project a total of 2 VCA (i.e. swine heterotopic hind limb) transplantations were performed using fully SLA mismatched hind limb donor, recipient pairs and a bone marrow donor that is MHC matched to recipients. The recipient animals were sensitized with SLA disparate donor skin grafts to achieve donor-specific presensitization. A desensitization protocol consisting of a 7day course of Fludarabine and 800cGy total body irradiation followed by MHC matched bone marrow transplantation was subsequently given 46 days post skin transplantation.

Due to continuing delays in animal availability from the breeder at Columbia University we were required to seek a 2nd No Cost Extension (NCE) for this project which we have submitted.

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

B.

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 2020	20 %	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.

Accomplishment of Goals

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

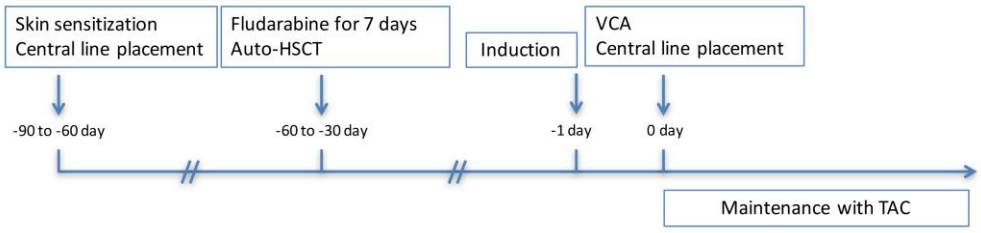

Figure 1: Treatment regimen of the recipient animal

Over the course of the past year, animal availability issues had created delays in this project. However, these issues did improve in the last 6 months, and allowed us to work on Major Task 3, group 5, as outlined in **Figure 1**. To complete this experiment fully SLA mismatched skin/hind limb donor Pig 24961 (AA), two recipient Pigs 24991 (GG) and 24992 (GG), and a bone marrow donor MHC matched to recipients, Pig 24990 (GG) were ordered. The sensitization phase of this experiment was initiated on 8/1/2019. Pigs 24991 and 24992 were taken to the OR for skin transplantation from donor pig 24962. A central venous access catheter was concurrently placed to allow for blood sampling for further assessment of the immune response via collection of serum and peripheral blood mononuclear cells (PBMCs). Both animals recovered well from the procedure.

Serum and PBMCs were harvested and stored at regular intervals 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-skin graft and post-skin graft donor specific responsiveness. As expected, immune responsiveness to the donor was maintained after sensitization by skin transplantation (**Figure 2**). Further, donor specific IgG formation (DSA) was demonstrated after skin sensitization by *in-vitro* flow cross-match (**Figure 3**).

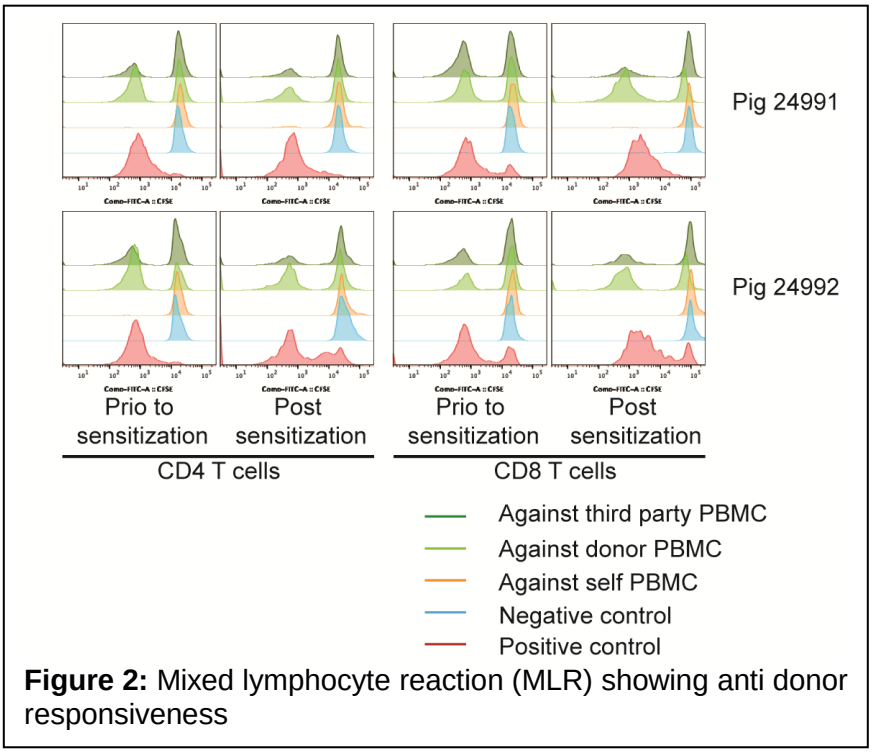

Figure 2: Mixed lymphocyte reaction (MLR) showing anti donor responsiveness

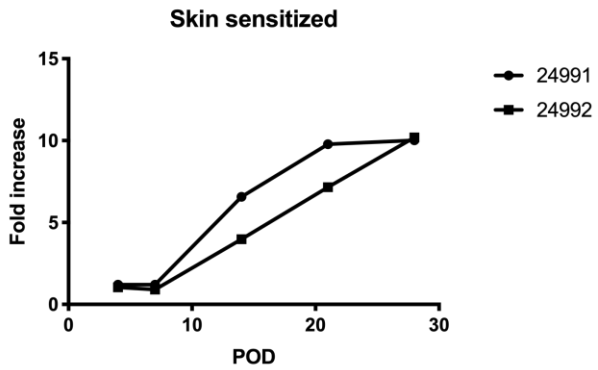

Figure 3: *In vitro* monitoring of serum DSA (IgG) formation after VCA in presensitized recipients

Once the presence of DSA was confirmed, attention could then be turned to the experimental desensitization protocol. Recipients animals were to receive Fludarabine for 7 days prior to myeloablative irradiation (800cGy) followed by MHC matched bone marrow transplantation. This was to be followed by hindlimb transplantation from the donor (to which recipient animals were previously sensitized). If the desensitization protocol were successful, allograft acceptance would be expected.

Animals tolerated Fludarabine treatment well. Animals then underwent myeloablative irradiation as planned. Animals were appropriately prophylaxed with antibiotic and ant-viral medication per veterinary advice. As myeloablation and resultant anemia was expected, animals were also transfused appropriately SLA matched blood.

However, by post-irradiation days 7 and 8, the animals began to exhibit lethargy, inappetence, and sequelae of coagulopathy. The animals were closely attended to by veterinary staff and give appropriate supportive treatment, but did not respond and by post-irradiation days 9-10 the animals had to be euthanized.

Due to this outcome, attention was directed at a full, post-mortem etiological work-up. Necropsy demonstrated severe neutropenia, radiation induced bone marrow suppression, followed GI toxicity and tissue damage, secondary bacterial translocation, and radiation-induced lung injury confirmed by veterinary pathologists.

While this was not the anticipated outcome, this work demonstrated a need to carefully re-evaluate our radiation dosing strategy as well as post bone marrow transplant supportive care. At this time, meetings are ongoing with on-site medical physics experts to adjust the irradiation dose and methodology and compare it against the published data. Bone marrow harvest techniques are again being revisited and perfected, and discussions with our veterinary colleagues have been carried out to further optimize post- irradiation supportive care. As such this careful re-evaluation is laying the groundwork for the rest of the group 5 animals required to complete this subtask.

C. Training and Professional Development

Large animal experiments are a critical component of validating proposed protocol prior to a potential application in the clinical VCA setting. Proposed regimen requires fine tuning to reduce radiation toxicity.

D. Result Dissemination

Nothing to report

E. Future plan

The start of the translational large animal experiments as outlined by the SOW under Task 3 were significantly delayed due to limited animal availability from the breeder at MGH/Columbia. However, over the past couple of months, we have made significant progress in satisfying our demands for the remainder of the project. To carefully address the specific needs of the experiments (SLA type, 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.
In light of irradiation toxicity noted with these animals, our team has taken and is continuing to take every possible precaution to prepare and optimize processes (irradiation, bone marrow harvest and processing) associated with the translational large animal experiments outlined by the SOW under Task 3. During the last 6 months, progress has been made with the first in-vivo pilot trials using a desensitization protocol. Based on these initial results, the remaining time and experiments during the NCE period will focus on further adapting and optimizing the components of the protocol to achieve VCA graft survival in the setting of sensitization.

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 reports, we have been experiencing significant delays with Major Task 3 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. Specific breeding efforts have been initiated and are now finally starting to yield animals that meet the demands for this study. We will continue to work with the breeder to advance these initiatives to assure continued access to the animals.

Additionally, as previously mentioned, radiation induced toxicity issues are being tackled with the help of Johns Hopkins radiation oncologists and medical physicists, and post radiation care strategies have been discussed at length with our veterinary colleagues.

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

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

Gerald Brandacher:
Closed 8/31/2019**:** Tissue Testing Technologies *Long Term Banking of Vascularized Composite Grafts using ice-free cryopreservation by vitrification and nano-warming tehcnologies*
Closed 9/17/19: DOD/Univ of Pittsburgh *Translational Cell Based Immunomodulatory Therapies in Vascularized Composite Allotransplantation.*
New 9/1/2019*:* DOD *A Novel Application of Normothermic Machine erfusion for Face Recovery to Reduce*
New 9/30/19: DOD *Ethical factors impacting patients decsions to pursue VCA*

C. Other organizations involved as partners

Nothing to Report