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AWARD NUMBER: W81XWH-16-2-0042

TITLE: Adult Tissue-Derived Stem Cells and Tolerance Induction in Nonhuman Primates for Vascularized Composite Allograft Transplantation

PRINCIPAL INVESTIGATOR: Eric A. Elster, MD

RECIPIENT: The Henry M. Jackson Foundation for the Advancement of Military Medicine
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REPORT DATE: October 2018

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2018	Annual	Sept 30,2017 - Sept 29,2018

4. TITLE AND SUBTITLE
Adult Tissue-Derived Stem Cells and Tolerance Induction in Nonhuman Primates for Vascularized Composite Allograft Transplantation-the VCA

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-2-0042

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

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5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER 2

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
820 Chandler Street, Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRMC

11. SPONSOR/MONITOR'S REPORT NUMBER(S) 2

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The utilization of adult derived adipose stem cells administration in composite tissue transplantation has shown positive results in multilineage T cell macrochimerism in rodents. This is a potential novel method of tolerance induction in humans, given the similarities to non-human primate models.

14. ABSTRACT
Amputations and unsalvageable injuries with devastating tissue loss are common in the combat wounded. Reconstructive transplantation in the civilian setting using vascular composite allotransplants (VCA) composed of multiple tissues (skin, muscle, nerve, bone) in combination with long-term multidrug immunosuppression has been encouraging. However, skin rejection remains a critical complication. We have demonstrated in a murine skin allograft transplantation model that human adipose-derived stromal cells (ASC) when used in concert with immunological conditioning support engraftment of limited numbers of donor bone marrow cells (dBMCs) across major histocompatibility complex (MHC) barriers, and lead to stable multilineage mixed-chimerism and skin allograft tolerance without the need for long-term immunosuppression.

Focus Areas: Immune Rejection-understanding mechanisms of immune rejection, immunomodulation approaches and mechanisms (e.g., tolerance induction, chimerism), and optimizing immunosuppressive drug regimens.

Objectives/Hypothesis: We realize that the implications and potential clinical benefit of the tolerance induction protocol to shown efficacy in a mouse model can only be validated mechanistically in established non-human primate models of allograft transplantation with long-term observations and evaluations. We hypothesize that ASCs+dBMC therapy may be a pro-tolerogenic cellular therapeutic displaying clinical efficacy for vascular composite allograft (VCA), solid organ, and hematopoietic stem cell transplant applications. This combination would allow for long term graft survival without the need for chronic immunosuppression and the resulting multitude of adverse effects associated with such agents.

Specific Aims: (1) To investigate whether ASCs augment chimerism and promote long-term VCA graft survival and (2) To determine whether ASC therapy allows for immunosuppression minimization and development of immunologic tolerance to VCA.

Study Design: We will use a non-human primate (NHP) model for facial vascular composite allografts (VCA) utilizing cynomolgus macaques that has demonstrated reproducible technical success over the last 10 years. Recipients will receive a VCA transplant on day 0 and then treated with the experimental immunosuppressive regimen. Non-myeloablative conditioning (anti-CD4/CD8 days 0-14, busulfan on day 5) and ASC + dBMC.

15. SUBJECT TERMS Vascularized composite allotransplantation (VCA), calcineurin inhibitors (CNI), adipose-derived stem cells (ASC), Non-human primate (NHP), tolerance					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	20	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Vascularized composite allotransplantation (VCA) have demonstrated graft survivals over a decade with conventional immunosuppression based on calcineurin inhibitors (CNI) have defined rates of renal failure, diabetes, and other side effects. Increasing the application of VCA to wounded military personnel depends on strategies that will safely extend graft survivals to many decades. This will be accomplished by immunosuppressive strategies that protect both the graft (decreased rejection) and the patient (decreased complications). Our studies investigate whether utilizing adipose derived stem cells (ASC) in these transplants can reduce or eliminate (tolerance) the need for immunosuppressive medications and associated toxicities. Ultimately, these experiments will answer the important question of whether the use of ASCs and low numbers of donor-derived bone marrow cells can promote the development of chimerism and tolerance, and represent an improved immunosuppressive strategy for VCA.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Vascularized composite allotransplantation (VCA), calcineurin inhibitors (CNI), adipose-derived stem cells (ASC), Non-human primate (NHP), tolerance

3. ACCOMPLISHMENTS:

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1 (100% completed)

Subtask 1: Submit documents for University of Maryland, Baltimore Institutional Animal Care and Use Committee (IACUC) approval. (completed 01OCT2016)

Milestone # 1 IACUC approval obtained (completed 19OCT2016)

Subtask 2: Submit documents for ACURO approval (approved Feb 22, 2017)

Milestone#2 ACURO approval obtained (100% completed)

Subtask 3: Contract/Order of 8 NHP for Aim 1 experiment

Milestone # 3 NHP received to Site 1 (100 % completed)

Subtask 4: Initiate 90 day quarantine, perform immunogenetic testing to select donor/recipient pairs for 4 experiments (100% completed).

Milestone #4 Completion of 90 day quarantine and selection of experimental pairs (100% completed)

Major Task 2 (65% completed)

Subtask 1: Surgical procedure of facial VCA transplant [(1 donor + 1 recipient) X 4 groups = 8 NHP total]

- Coordination and delivery of prepared cell products to UMB
- 30-day follow-up including clinical course and in vitro assays

Milestone #5 Completion of Aim 1 experiments

Subtask 2: Assess primary outcomes of 30-day graft survival and chimerism

- Data analysis and interpretation of diagnostic assays performed at UMB and USUHS including cell products, flow cytometry, laboratory data, graft biopsies

Milestone #6 Completion of chimerism studies and laboratory assays

Major Task 3 (65% completed)

Subtask 1: Immunosuppression weaning to low-level tacrolimus monotherapy (Day 31-90)

- Follow-up including clinical course (rejection, laboratory data, animal health assessments) and chimerism assays (flow cytometry data)

Milestone #7 Completion of immunosuppression weaning

Subtask 2: Stop immunosuppression to test for development of immunologic tolerance

- Follow-up including clinical course (rejection, laboratory data, animal health assessments) and chimerism assays (flow cytometry data) and data analysis to determine success of approach

Milestone #7 Completion of immunosuppression cessation

Subtask 3: Donor skin and 3rd party skin grafting to assess for donor-specific tolerance

Milestone #8 Completion of skin graft and immunologic assays

What was accomplished under these goals?

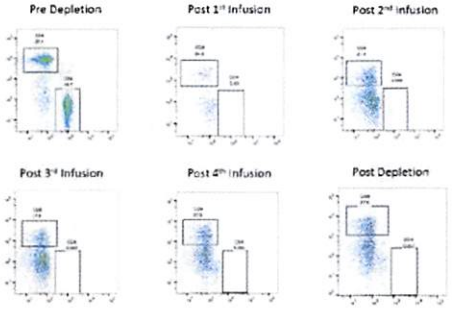
Accomplishment 3: We have continued to optimize methods to isolate, *ex vivo* propagate, functionally characterize, and cryopreserve (biobank) non-human primate adipose-derived stem cells (ASCs) derived from non-human primates. Multiple samples of donor adipose tissue was harvested from euthanized non-human primates housed the University of Maryland and the Tulane University National Primate Research Center were used for ASC isolation, ex vivo expansion characterization and cryopreservation biobanking for use in our vascular composite allograft transplant studies. It is estimated that > 600 million cells have been biobanked. Each VCA transplant animal recipient will require approximately 100 million culture expanded ASCs.

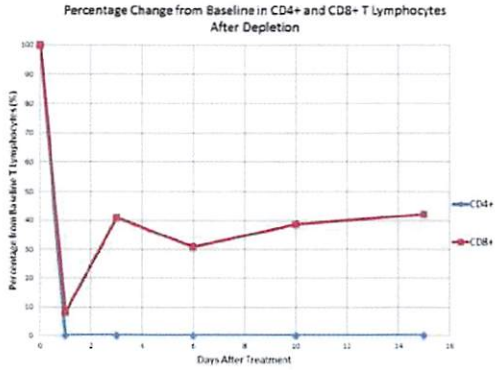
Results: The past funding period is noteworthy for the following accomplishments: (1) we have tested a proprietary combination of antibiotics/antimycotics (LaCell Swamp Juice™) to ex vivo expand non-human primate (NHP) ASCs without evidence of bacterial or yeast growth; (2) ASCs have been stained for classic cell surface markers to confirm an undifferentiated multipotent stromal cell phenotype. In addition, experiments were conducted to confirm that the isolated NHP ASCs are highly multipotent capable of differentiating into adipocytic, chondrocytic and osteocytic lineages on suitable stimulation

Accomplishment 2: In vivo assessment of the immunodepletion strategy in normal NHPs. We have identified the need to test depletional antibodies and busulfan in 1-2 NHP. This is necessary to confirm dosing and efficacy of the proposed drugs. Potential toxicities needed to be identified prior to commencing with the experimental group. In order to test these reagents in vivo, additional IACUC amendment and ACURO approval was necessary. IACUC approval was received from University of Maryland on 5 OCT 2017. ACURO submission was 3NOV2017.

Specific objectives: Two cynomolgus macaques received depletional therapies with CD4 and CD8 monoclonal antibodies and busulfan infusion in accordance with the study protocol to determine the tolerability of the treatment regimen and the amount of lymphocyte depletion.

Results: The first animal demonstrated good tolerability of the treatment regimen. CD4 populations were nearly completely depleted in peripheral blood as analyzed by flow cytometry. CD8 populations were only depleted by 70%. A second animal was treated with an increased intensity of anti-CD8 antibody and demonstrated depletion of 65%. The remaining non-depleted CD8 population has been hypothesized to be part of a memory T cell compartment that may have resistance to depletion with the utilized CD8 monoclonal antibody.





Major Accomplishment 3: Tolerance induction after Adipose Stem Cell Derived Infusion in VCA facial transplantation.

Specific objectives: Three cynomolgus macaques received heterotopic facial subunit transplant per Figure 1 & 2 (3 donor animals, 3 recipient animals) on postoperative day 0. CD4 and CD8 monoclonal antibodies (50 mg/kg) were administered on postoperative day 0, 2, 5, 7, and 14 as well as a non-myeloablative dose on postoperative day 5 of Busulfan (5 mg/kg). On postoperative day 7 adipose derived stem cells (20 x 10^6 cells/kg) and vertebral bone marrow (20 x 10^6 cells/kg) were infused. Chimerism and clinical evidence of rejection were followed postoperatively with serial biopsies and peripheral blood samples.

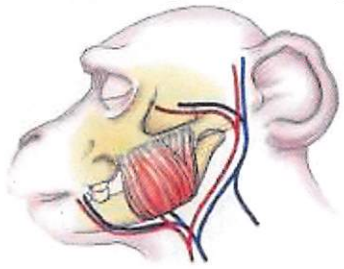


Fig. 1. Donor composite facial graft (above) and schematic (below) outlines. The osteomyocutaneous facial segment was based on the common carotid artery and both jugular veins, and included the facial, transverse facial, and superficial temporal arteries.

Fig. 2. Intraoperative photographs (above) and schematic drawings (below) of facial subunit depicting bone, muscle, and skin; the common carotid artery; and the internal and external jugular veins.

Results: 23JAN2018 Animal G31V underwent transplantation and subsequent ASC/VBM transfusion 30JAN2018. The graft progressed per Figure 3, with clinical evidence of early rejection POD 31.Endpoint was reached POD 37 with clinical and pathologic evidence of rejection. Figure 4 illustrates Grade 4 rejection with arteritis, moderate-severe inflammation within the muscle tissue and necrosis.

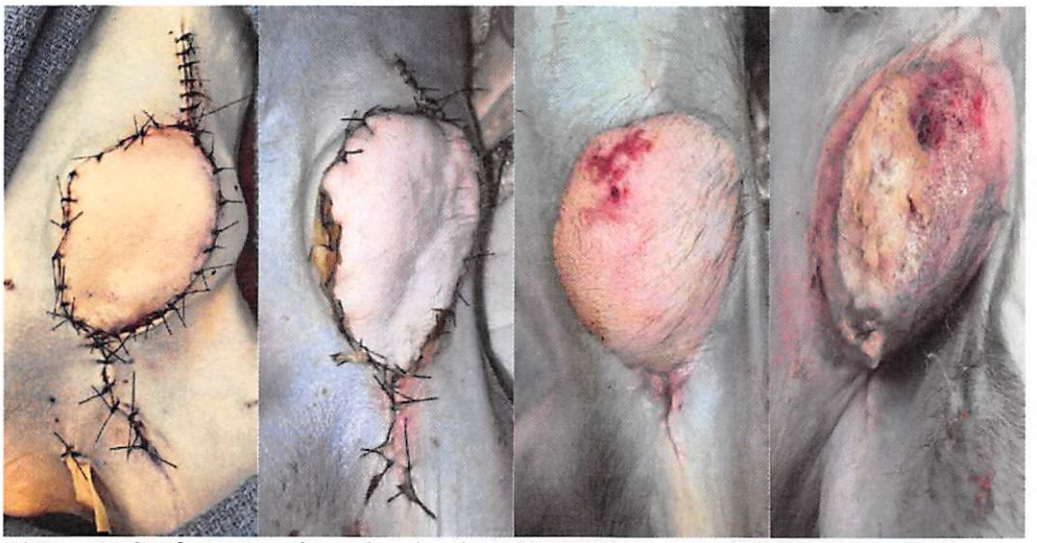


Figure 3. Graft progression of Animal G31V on POD 0, 10, 31, and 37.

CD4 and CD8 levels were determined using flow cytometry after POD 0,2,5,7, and 14 infusions. Figure 5 shows the percentage change from baseline in T lymphocytes.

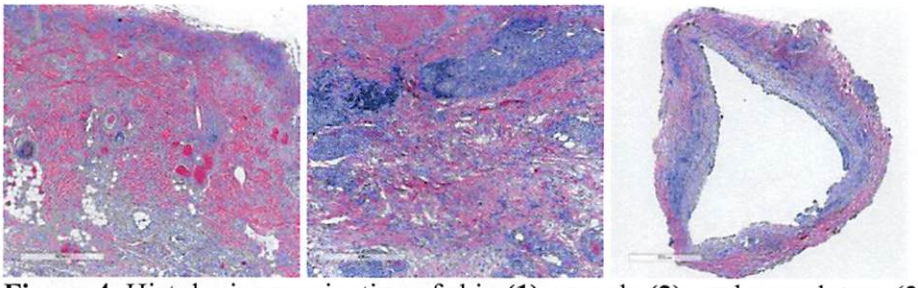


Figure 4. Histologic examination of skin **(1)**, muscle **(2)**, and vasculature **(3)** POD 37.

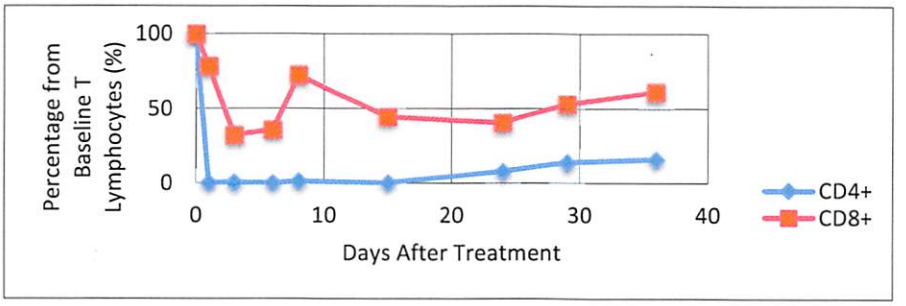


Figure 5. Percentage change from baseline in $CD4^+$ and $CD8^+$ T Lymphocytes after depletion.

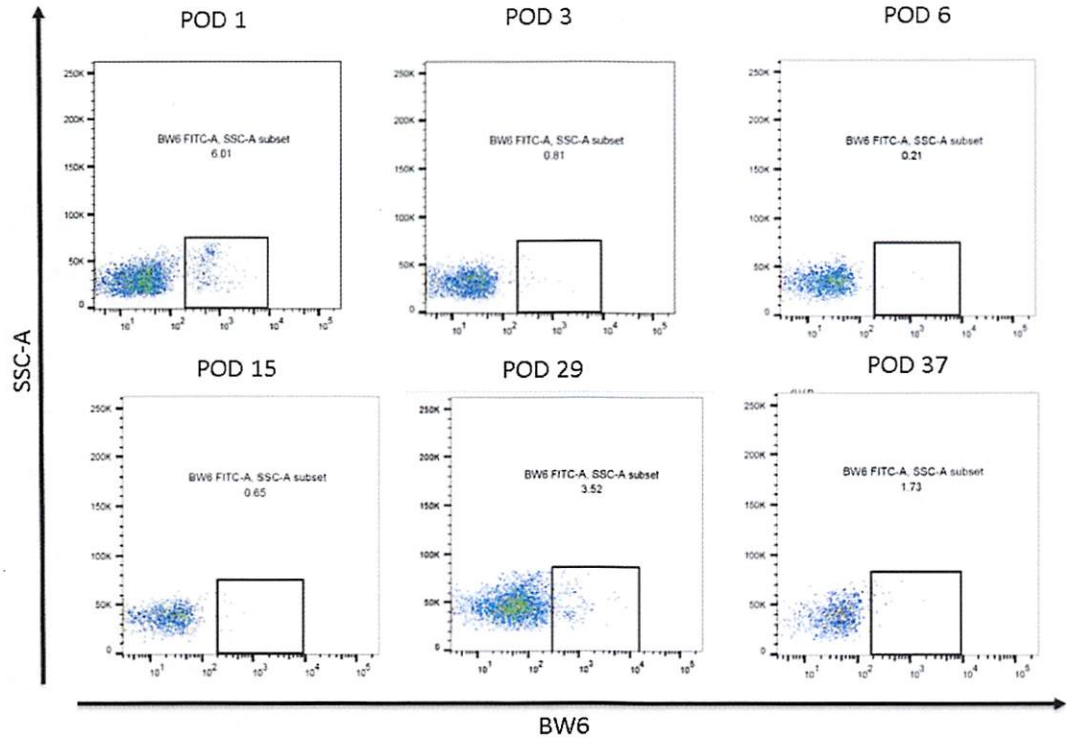


Figure 6. Animal G31V flow cytometry analysis of chimerism in peripheral blood with 6.01% on POD 1, 0.81% on POD 3, 0.21% on POD 6, 0.65% on POD 15, 3.52% on POD 29, and 1.73% on POD 37.

For experiment two Animal G21F underwent transplantation 20MAR2018 and subsequent ASC/VBM transfusion 27MAR2018. Graft progression is illustrated in Figure 7, with endpoint at POD-78.

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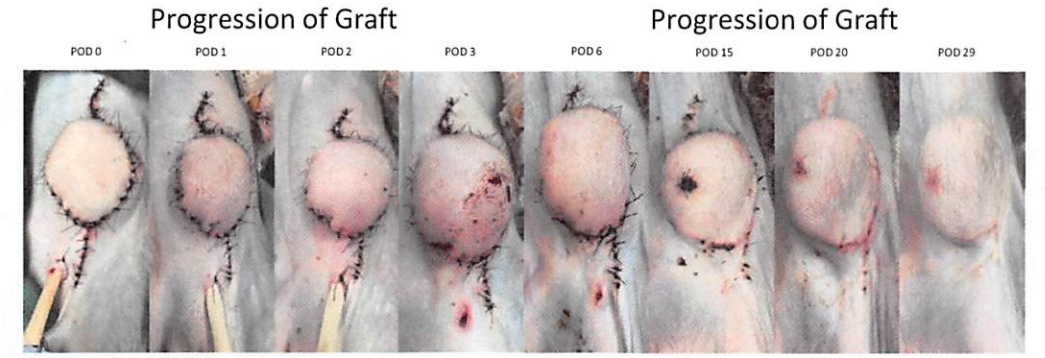
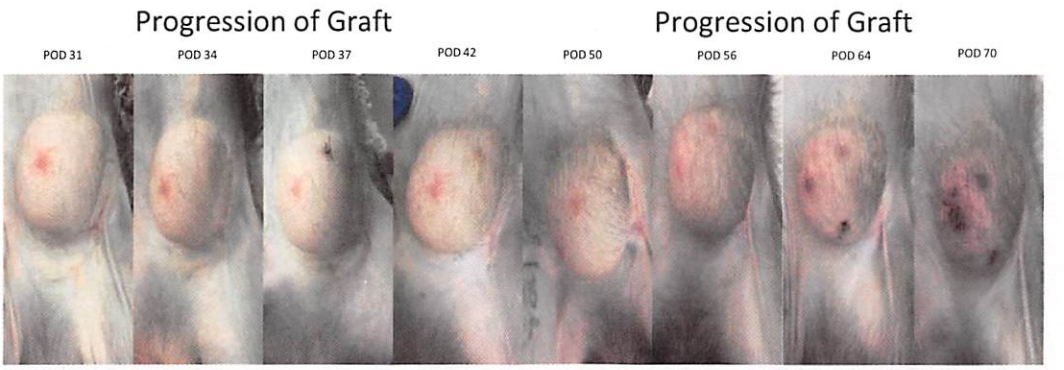
Clinical Log

- POD 1 10 mL blood transfusion
- POD 2 CD 4/8 administered
- POD 7 ASC/VBM Infusion, bleeding from biopsy site
- POD 14 CD 4/8 Infusion
- POD 30 FK target weaned to 10-15 ng/mL
- POD 43 skirt/line removed
- POD 64 biopsy taken
- POD 70 eschar developing
- POD 77 complete eschar





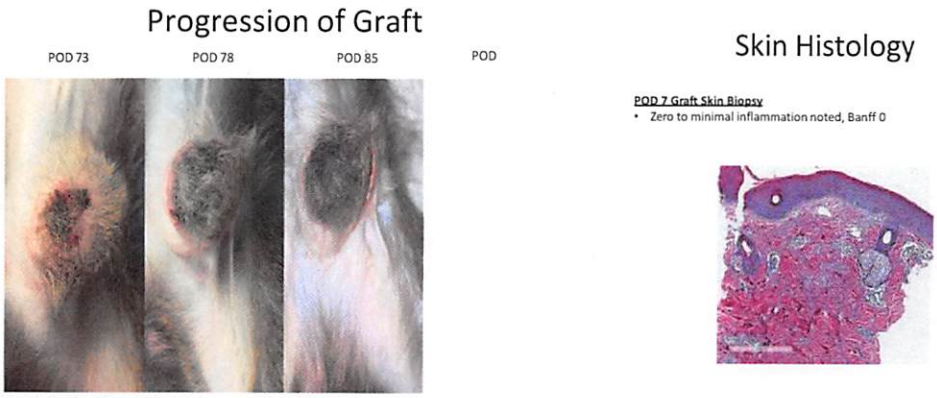


Figure 7. Graft progression on POD 0-85 of animal G21F.

G21F Peripheral Blood Leukocyte Chimerism

POD 1: 1.84 %
POD 3: 3.65 %
POD 6: 1.98 %
POD 8: 1.57 %
POD 15: 13.48 %
POD 22: 1.24 %
POD 29: 1.37 %
POD 37: 0 %
POD 42: 0 %
POD 50: 0 %
POD 56: 0 %
POD 64: 0 %
POD 73: 0 %
POD 78: 0 %

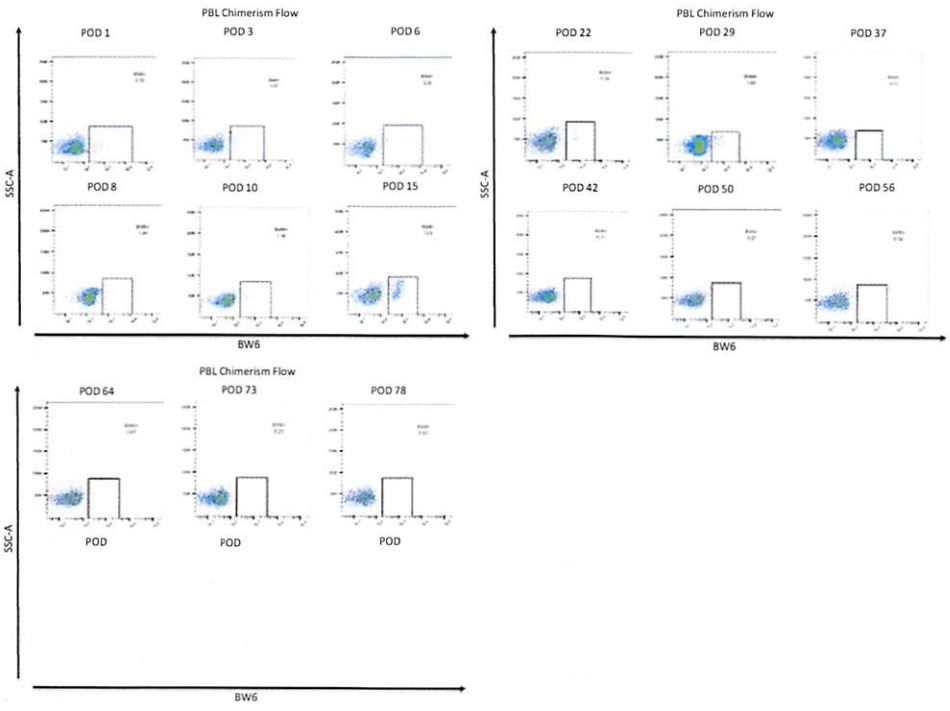


Figure 8. Animal G21F flow cytometry analysis of chimerism in peripheral blood with 1.84% on POD 1, 3.65% on POD 3, 1.98% on POD 6, 1.57% on POD 8, 13.48% on POD 15, and 1.24 on POD 15.

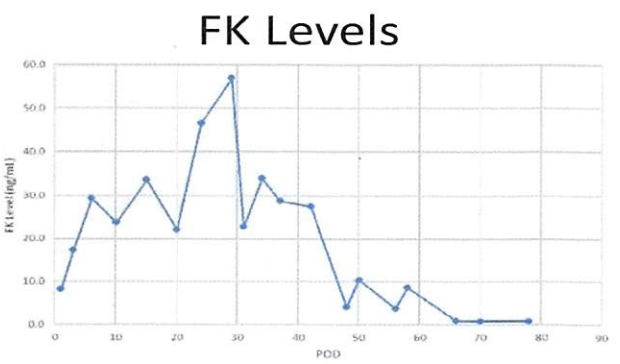


Figure 9

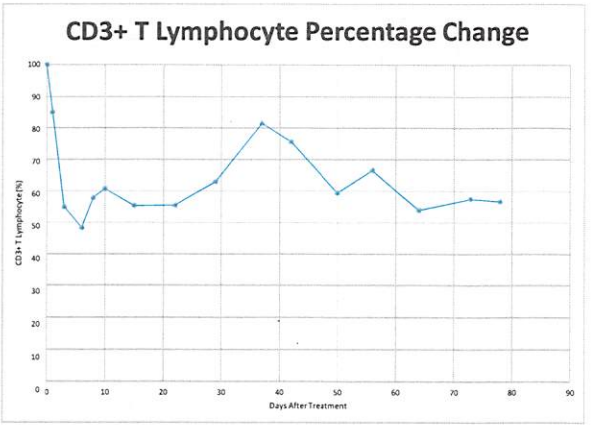


Figure 10

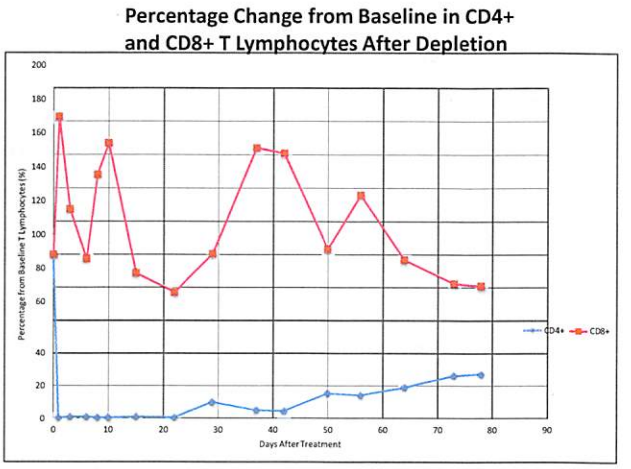


Figure 11

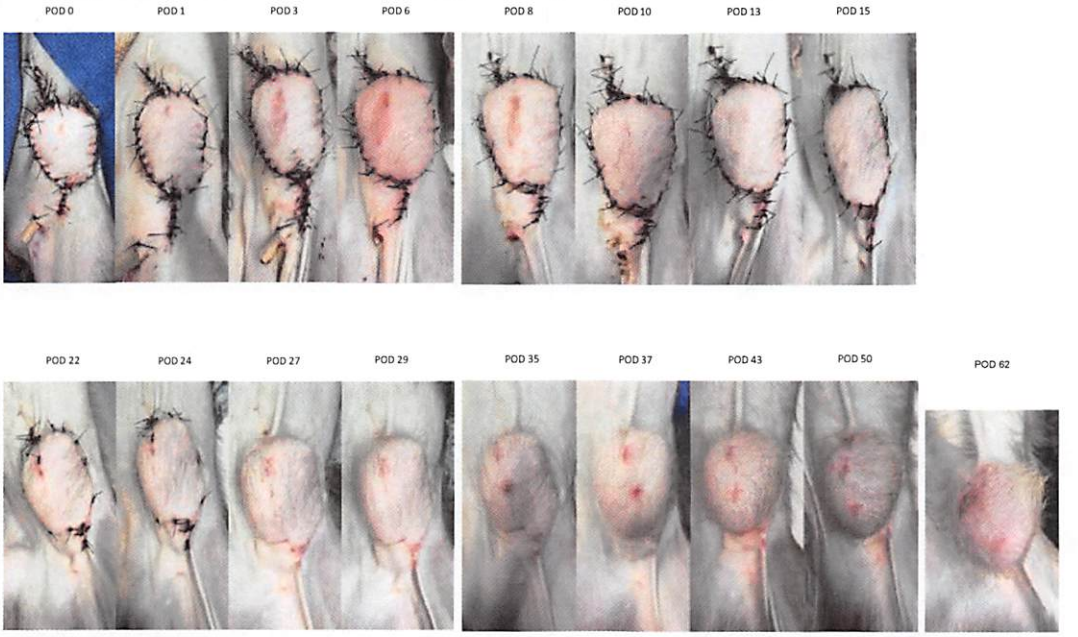
Figures 9, 10 and 11. Animal G21F serum FK levels, and circulating $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes counts. Graft rejection in this animal correlated with the tampering FK (~ days 40-50 post VCA transplantation) and the rise in circulating $CD4^+$ cells (~ POD 50-77)

For experiment three Animal G50G underwent transplantation 31July2018 and subsequent ASC/VBM transfusion 8AUG2018. Graft progression is illustrated in Figure 12. As of 4OCT2018 (POD-65) the graft was viable with potential early visible signs of rejection (cutaneous erythema) noted on POD-62.

Clinical Log

- POD 0 15 mL transfusion
- POD 3 abrasion noted to middle/upper portion graft
- POD 7 ASC/dVBM infusion tolerated well
- POD 8 abrasion healing
- POD 22 neutropenic
- POD 27 neutropenia improving
- POD 43 graft swelling
- POD 45 swelling stabilized
- POD 50 superior aspect abrasion

Progression of VCA graft (Figure 12)



G50G Chimerism

POD 1: 4.25% chimerism in blood
POD 3: 1.57% chimerism in blood
POD 6: 1.78% chimerism in blood
POD 8: 1.86% chimerism in blood
POD 22: 26% chimerism in blood
POD 29: 7.81% chimerism in blood
POD 37: 14.6% chimerism in blood
POD 43: 0.97% chimerism in blood

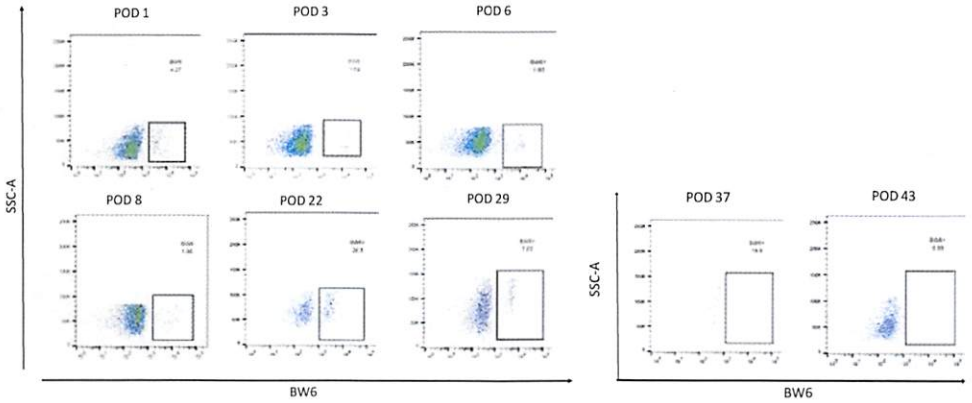
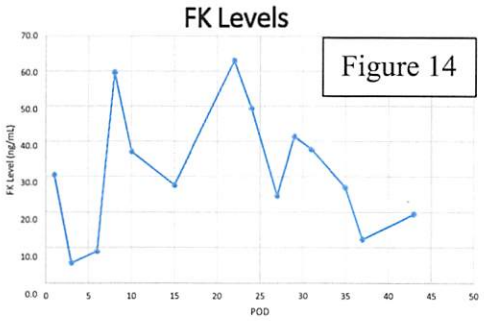
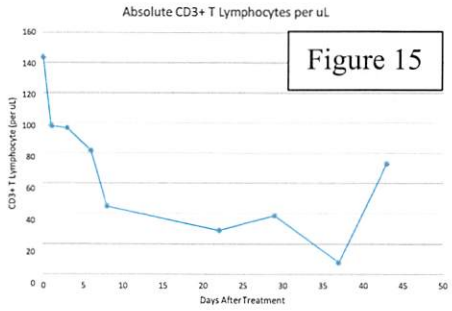
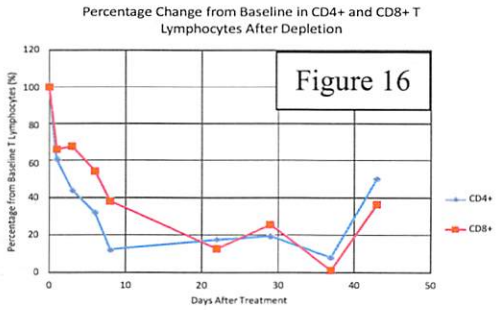


Figure 13. Animal G508F flow cytometry analysis of chimerism in peripheral blood with 4.25% on POD 1, 1.57% on POD 3, 1.78% on POD 6, 1.86% on POD 8, 26% on POD 22, 7.81% POD 29, 14.6% on POD 37 and 0.97% on POD 43.







Figures 14, 15 and 16. Animal G508 serum FK levels, and circulating $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes counts. Potential early visible signs of rejection (cutaneous erythema) at POD-62 in this animal correlated with the tampering FK (~ days 40-50 post VCA transplantation), loss of PB chimerism and the rise in circulating $CD4^+$ cells (~ POD 43; Figure 16).

Summary: Remarkable findings related to short-term/transient donor cell chimerism and prolonged graft survival have been observed in two of the three VCA transplanted NHPs conducted. Early evidence of graft rejection in these animal correlated with the tampering of FK (~ days 40-50 post VCA transplantation), the loss of peripheral blood mixed chimerism and the rise in circulating $CD4^+$ cells (~ POD 50-77). The sponsor has granted our team a 1-year no-cost extension to commence with the 4th NHP in the 1st Qrt of FY19. We project that all the data analyses and final report will be completed by the end of the 2nd Qrt. Overall, significant progress has made in this pilot study-Idea Award. The experimental strategy and results are extremely promising towards extending VCA graft survival and allograft tolerance using a durable/stable chimeric approach. We are in the process of seeking follow-on funding to test in this model (1) a more effective anti-CD8 depletional antibody; (2) the timing of multiple infusion of ASCs post transplantation; and (3) a larger number of animals with VCA transplants in order to perform more in-depth mechanistic assessments at the systemic, tissue, cellular, proteomic and genomic levels.

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

The laboratory has been very active in the training of research fellows and young faculty. During the period of funding support, three research fellows participated in funded studies. One fellow has returned to completing surgical training with intention to apply for clinical fellowships in plastic and reconstructive surgery.

Our laboratory's overall record of accomplishment overall has resulted in training 12 graduate and post-doctoral fellows and 8 undergraduates in transplant immunology and reconstructive transplantation. They have been trained in surgical techniques of transplantation in animal models, large animal handling, immunological lab techniques, assistance with care and evaluation of clinical VCA patients, as well as the logistics of running a large animal lab. Four of these fellows have completed plastic surgery fellowships and three have academic appointments as faculty in plastic and reconstructive surgery. Two prior fellows are completing clinical fellowships in cardiothoracic surgery and plastic surgery.

How were the results disseminated to communities of interest?

Nothing to Report

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

The sponsor has granted our team a 1-year no-cost extension to commence with the 4^{th} NHP in the 1^{st} Qrt of FY19. We project that all the data analyses and final report will be completed by the end of the 2^{nd} Qrt.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

The utilization of adult derived adipose stem cells administration in composite tissue transplantation has shown positive results in multilineage T cell macrochimerism in rodents. This is a potential novel method of tolerance induction in humans, given the similarities to non-human primate models.

What was the impact on society beyond science and technology?

Immunosuppression required at time of transplantation, such as calcineurin inhibitors, can lead to a number of side effects, including renal failure, infection, and malignancy. These present significant health risks, mortalities, and expenses to patients and their families, hospital systems, and society at large. Establishing a tolerance regimen has the potential to provide longer lasting and more cost efficient method to protect transplanted organs and tissues.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

We have identified the need to test depletional antibodies and busulfan in 1-2 NHP. This is necessary to confirm dosing and efficacy of the proposed drugs. Potential toxicities needed to be identified prior to commencing with the experimental group.

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

In order to test these reagents in vivo, additional IACUC amendment and ACURO approval was necessary. IACUC approval was received from University of Maryland on 5OCT2017. ACURO submission was approved 7NOV2017. The need to test the reagents in 1-2 NHP have affected expenditures with drug and animal purchasing.

The major change to the approach is that we will now consider expansion of the ASC for extended periods of time and through multiple successive passages. While we initially had expected to obtain a sufficient number of ASC directly from a single passage of the adipose tissue-derived SVF cells, we have recognized that this is not tenable due to the small amount of subcutaneous adipose tissue available in most non-human primates.

Changes that had a significant impact on expenditures

The major problem that we have faced has been contamination of specimens, possibly at the time of harvest, in transit, and/or during the culture expansion period. We have addressed this by developing a more potent cocktail of bacterial and fungal inhibitors which is now being used as a culture medium supplement. Additionally, concentrated antibiotics and antimycotics are being added to the medium during the shipping period.

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

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals.

IACUC amendment to test CD4, CD8, and busulfan in up to 2 animals to test drug efficacy. Approved 5OCT 2017. ACURO approval on 3NOV2017.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Eric A. Elster, MD
Project Role: Principal Investigator
Nearest person month worked: 3
Contribution to Project: Provides oversight of the entire project development and implementation of al policies, procedures, and processes.

Name: Rolf Barth, PhD
Project Role: Co-Principal Investigator
Nearest person month worked: 5
Contribution to Project: Provides oversight of the entire project development, serves to ensure Completion of the specific aims on the schedule proposed and scientific quality control for animal study analysis, and troubleshoots technical issues that may arise in the day-to-day management of the project.

Name: Thomas A. Davis, PhD
Project Role: Co-Investigator
Nearest person month worked: 3
Contribution to Project: Assists with project development, serves to ensure completion of the specific aims on the schedule proposed and scientific quality control for animal study analysis, and troubleshoots technical issues that may arise in the day-to-day management of the project.

Name: Jeffrey Gimble, MD, PhD
Project Role: Research Collaborator
Nearest person month worked: 5
Contribution to Project: Isolation, ex vivo expansion, functional characterization and the cryopreservation of non-human primate adipose-derived stem cells.

Name: Nicole Schockcor, MD
Project Role: General Surgery Resident/Research Fellow
Nearest person month worked: 5
Contribution to Project: Direct role in data acquisition, animal care and experimental organization.

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.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Not Applicable.

Adult Tissue-Derived Stem Cells and Tolerance Induction in Nonhuman Primates for Vascularized Composite Allograft Transplantation

Congressionally Directed Medical Research Programs - W81XWH-16-2-0042

PI: Eric Elster, Co-PI Rolf N. Barth **Org:** The Henry Jackson Fdn. for the Advancement of Mil. Med., Inc.

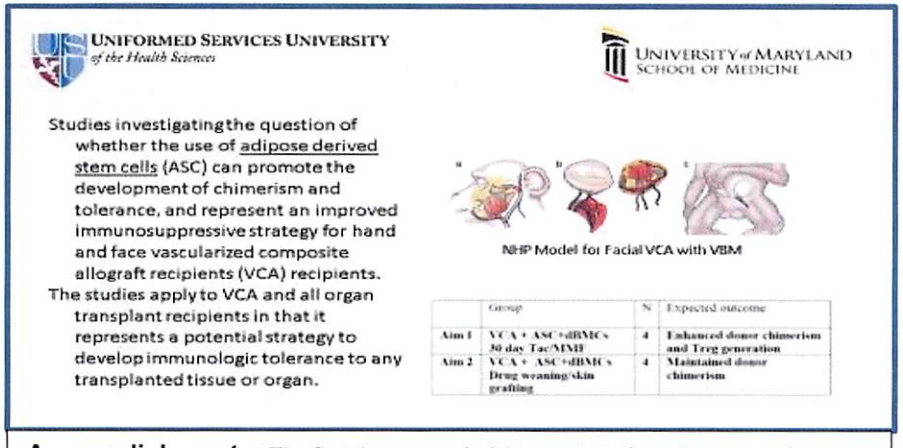
Work Perform at: Uniformed Services Univ. of the Health Sciences/Univ. of Maryland **Award Amount:** 448,415

Study/Product Aim(s)

- Our studies investigate whether utilizing adipose derived stem cells (ASC) in hand/face vascularized composite allograft (VCA) transplants can reduce or eliminate (tolerance) the need for immunosuppressive medications and associated toxicities in service members who would benefit from these reconstructive approaches to devastating facial and limb injuries.
    - Aim 1. To investigate whether ASCs augment chimerism and promote long-term VCA graft survival.
    - Aim 2. To determine whether ASC therapy allows for immunosuppression minimization and development of immunologic tolerance to VCA.

Approach

Non-human primate (NHP) experiments in our established model of facial VCA to examine the ability of ASC establish chimerism, promote graft survival, and establish immunologic tolerance.



Accomplishments: The first three maxofacial transplants have been completed ASC/VBM transfusion. The third VCA transplant is on-going with early visual signs of rejection at POD-62. The 4th is scheduled for Oct/Nov 2018. Great progress and remarkable findings related to short-term donor cell chimerism and prolonged graft survival have been observed.

Timeline and Cost

Activities FY	16	17	18
ASCs augment chimerism in NHP facial VCA			
ASC therapy allows for immunosuppressive therapy reduction and withdrawal in NHP facial VCA			
Estimated Budget ($448K)	**0**	**280**	**168**

Updated: October 5, 2018

Milestones/Goals

FY16 Goals

- ✓ Approved UMB IACUC
- ✓ Approved DOD ACURO

FY17 Goals

- ✓ NHP animals screened, selected, and delivered to UMB site
- ✓ Test the immunodepletion/condition strategy in normal NHPs
- ✓ Isolate, ex vivo expand , and functionally characterize NHPs ASCs
- ✓ Develop a biobank of NHP ASCs for in vivo transplantation

FY18 Goals

- ❑ completed four partial maxofacial VCA transplants with up to 30-day follow-up
- ✓ Wean graft transplanted NHPs to low level immunosuppression for pending cessation after facial transplant
- ❑ Complete skin grafts and determination of tolerance.
- ❑ Complete follow up data analysis of all NHP VCA experiments

Budget Expenditure to Date:

Actual Expenditure: $270,328.96