

AWARD NUMBER: W81XWH-20-1-0943

TITLE: Effect of Peritransplant C3d Blockade and Ischemia on
Chronic Rejection and Vasculopathy in an Experimental OMC Flap
Model of VCA

PRINCIPAL INVESTIGATOR: Christina L. Kaufman PhD

CONTRACTING ORGANIZATION: University of Louisville Research Foundation

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14. ABSTRACT Based on observations that the complement protein C3d plays a pivotal role in the regulation of cell mediated immunity, we are hypothesizing that blockade of this protein early in the post-transplant period may mitigate rejection, and protect the graft from ischemia reperfusion injury (IRI). We are using our allogeneic rat VCA model to determine where C3d and its receptor CD21 binds in the allograft, and how that binding differs in titrated immunosuppression models designed to mimic severe acute cellular rejection (ACR), mild ACR and chronic rejection (CR). Interestingly, C3d binds in ACR, and binds first to structures within the hair follicle in the skin as well as vessels. CD21 expression in the graft increases significantly after C3d binding is evident. We have also started the cold ischemia groups and are assessing how IRI influences C3d and CD21 expression. We are producing bulk antibody from a cell line with an IgG-CD21 dimer and expect to start in vivo blockade experiments in the first quarter of year 2. The overall goal of the project is to reduce the amount of immunosuppression required to maintain VCA allografts, reducing the risk/benefit ratio and allowing wider access to VCA transplantation.					
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1. INTRODUCTION:

Based on observations that the complement protein C3d plays a pivotal role in the regulation of cell mediated immunity, we are hypothesizing that blockade of this protein early in the post-transplant period may mitigate rejection, and protect the graft from ischemia reperfusion injury. We will extend studies of our allogeneic rat VCA model to determine where C3d and its receptor CD21 binds in the allograft, and then the effect of blockade of C3d by a CD21-dimer antibody on the development of acute and chronic rejection. Of note we have titrated the level of timing of tacrolimus immunosuppression to mimic clinical observations seen with respect to acute cellular rejection (ACR), vasculopathy, and chronic rejection of the skin. These models will be used to determine if C3d blockade can protect the graft from increased cold ischemia injury. Finally, we will test if the use of C3d blockade will allow the use of lower amounts of immunosuppression (tacrolimus) to prevent acute and chronic rejection. The overall goal of the project is to reduce the amount of immunosuppression required to maintain VCA allografts, reducing the risk/benefit ratio and allowing more amputees and patients with catastrophic tissue loss access to the life changing therapeutic option of VCA transplantation

2. KEYWORDS:

VCA, complement, graft rejection, reduction of immunosuppression, experimental model

3. ACCOMPLISHMENTS:

○ What were the major goals of the project?

Major Task	Timeline	Percent Completed
Major Task 1 Submit IACUC documents for local IACUC review and ACURO approval	Month 1-3	100%
Major Task 2 –Perform rat OMC flap transplants in Groups 1-4, in syngeneic and allogeneic acute rejection and chronic rejection models	Month 3-9	100%
Major Task 3 –Perform rat OMC flap transplants in Groups 5-12, test how cold ischemia and/or C3d blockade change pathology of the graft.	Month 9-18	40%

Major Task 4 use a model of acute rejection and reduce the dose of tacrolimus in subsequent groups with and without C3d blockade to see if C3d blockade allows a lower dose of tacrolimus that is sufficient to maintain the graft	Month 18-23	0%
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○ **What was accomplished under these goals?**

Major Task 1: We are pleased to report that both local IACUC as well as ACURO approval was obtained by 9/22/20, prior to the award start date of 9/30/20.

Major Task 2: As outlined in our quarterly reports, we have had ongoing technical issues with the rat surgery in that were addressed and resolved in year 1 of the project, but resurfaced again when we starting doing the groups with additional cold ischemia in year 2 of the project. With an additional two hours of cold ischemia we had substantial bleeding and lost multiple animals. We spent a significant amount of time consulting with surgery colleagues at UofL and at other centers as well as testing different doses of heparin and then increased the amount of flush that was used before and after the donor grafts were stored at 4C for two hours of cold ischemia.

Table 1 summarizes the groups which have been transplanted to date, as well as the remaining groups to be done. Note that when the project budget was submitted in 2019, Brown Norway rats were available for approximately \$80. Animal costs skyrocketed during and following the pandemic and we are now paying 3 to 4 times that for a single rat, not counting shipping and handling fees. As such budget constraints have reduced our target N for each group to 4-6 animals. We are confident that the experimental objectives can still be achieved with this number of animals.

Group #	Transplant	Strain Comb.	Rejection model*	At least 4 animals transplanted?	Comments
1	Syngeneic OMC flap	Lew > Lew	No tacrolimus	Yes	
2	Syngeneic OMC flap	Lew > Lew	Standard tacrolimus	Yes	
3	Allogeneic OMC flap	BN > Lew	Standard tacrolimus	Yes	
4	Allogeneic OMC flap	BN > Lew	Suboptimal tacrolimus	Yes	
5	Syngeneic OMC flap	Lew > Lew	2 hours of cold ischemia time and No tacrolimus	Yes	
6	Syngeneic OMC flap	Lew > Lew	2 hours of cold ischemia time Standard tacrolimus	No	Our microsurgeon took another position
7	Syngeneic OMC flap With C3d blockadge	Lew > Lew	No tacrolimus	No	
8	Syngeneic OMC flap With C3d blockadge	Lew > Lew	No tacrolimus	No	
9	Allogeneic OMC flap	BN > Lew	2 hours of cold ischemia time Standard tacrolimus	Yes	

10	Allogeneic OMC flap With C3d blockade	BN > Lew	Standard tacrolimus	No	
11	Allogeneic OMC flap With C3d blockade	BN > Lew	2 hours of cold ischemia time Standard tacrolimus	No	
12	Allogeneic OMC flap WITH C3d blockade	BN > Lew	Suboptimal tacrolimus	No	

*Standard Immunosuppression is 2mg/kg/day until day 12, then 0.5mg/kg/day

Suboptimal Immunosuppression is 2mg/kg/day until day 12, then 0.5mg/kg until day 28, then 0.25mg/kg/day

In the tissue samples stained to date, animals receiving an allogeneic graft have demonstrated localized C3d deposition, with more diffuse staining at week 2 post-transplant. Similar to the syngeneic group, there is minimal C3d deposition in the day 0 sample but week 2 and week 4 skin samples from Group 3 and 4 analyzed to date shows widespread C3d deposition. We were expecting localization of C3d deposition in vessels, but it appears the adnexal structures (hair follicles, sebaceous glands) are also being targeted. This may prove to be a very interesting finding, as from our H&E studies in this model, we routinely see loss of adnexal structures in the donor skin of the graft with sparing of these same structures in adjacent recipient skin.

While the standard immunosuppression animals do not demonstrate severe ACR, we know that mild ACR is ongoing, and animals can show mild vasculopathy of the femoral artery. (These observations were submitted to the Journal of Surgical Research, which is in the final stages of review, and we were asked for images for the cover and once the final revisions are accepted the manuscript will be one of the three featured articles.) As we know the hair follicle is a target of chronic rejection in both rats and humans, this finding suggests that C3d staining may also serve as a marker of chronic rejection.

We performed a similar analysis of CD21 staining. Skin biopsies from syngeneic transplants at the same timepoint showed no staining, while standard immunosuppression models showed diffuse staining and acute ACR animals showed intense staining at week 2 suggesting an influx of B cells to the area. While standard immunosuppression animals showed some staining at week 1 and week 2, the level of CD21 staining correlated with the mild rejection that does eventually occur in these animals.

We currently have a tissue block repository of more than 700 samples (and growing) from the animals transplanted to date, including skin biopsies, and explant samples of skin, spleen, distal artery, proximal contralateral artery, arterial anastomosis, conjunction of donor/recipient skin, and donor skin.

Unfortunately, we had two major setbacks and problems with immunohistochemistry in year 2 of the grant. The major issue was that we had sent the CR2 IgG dimer hybridoma that was supplied to us by our colleagues at the University of Michigan subaward. This construct has a dimer of CR2 attached to an IgG1, and effectively blocks interaction of C3d with cells expressing the CR2 receptor (primarily B cells and follicular dendritic cells, as well as subset of T cells). The volume of antibody that will need to be given to the rats at the time of transplant in the C3d blockade is fairly large, at 8.8 mg. As we discussed in previous quarterly reports, we did contact multiple commercial vendors and got quotes to grow hybridoma supernatants and isolate, purify and perform infectious disease testing for use in this study. We chose Precision Antibody of Columbia MD, and they will grow the hybridomas, perform mycoplasma testing, do the pilot production and purification and provide us with 500 to 1 mg of affinity purified antibody, which will be sufficient. We expected to have this antibody for use in blockade experiments during the 1st quarter of year 2 of the project.

After more than 6 months, and over \$5,000 in fees, the company reported that the hybridoma was a poor producer. Additional conversations determined that the company had failed to culture the hybridoma with the antibiotic G418, which selected for the cells with the CR2dimerIgG antibody insert. After some additional unproductive conversations with the company, we decided to grow the hybridoma in Louisville and isolate the antibody here.

Dr. Cascalho had one of her post docs thaw a vial, and culture it in the presence of G418 to select for the hybridomas with the CR2IgG insert. Supernatant from the re-cultured cell line was tested to confirm it was making antibody that reacted with C3d. It did, and one vial of this hybridoma lot was sent to Louisville along with 28 ug of antibody that was isolated.

The second major set back was that our microsurgeon received an offer as a pharmaceutical representative and left the lab. At that time we still had one group of the cold ischemia controls to transplant, and all of the groups with C3d blocking as we did not have sufficient quantities of the antibody to do the blocking experiments.

In the 3rd quarter of year 2 we submitted a request for a NCE and a re-budget of funds that would allow us to hire a part time microsurgeon in year 3 of the grant. This request and re-budget was approved. It took some time to find someone with the skills to do the microsurgery and be willing to work on a part time basis. Happily, we were able to hire Dr. Ran Rutenberg, who started in the first quarter of year 3, and he is progressing rapidly in his training, and has mastered the anastomosis of the femoral vessels, arguably the most difficult part of the procedure. Dr. Rutenberg is a Emergency Medicine resident from Israel, here in Louisville for the next year as his wife is doing a fellowship at UofL. He has excellent hands for microsurgery and we are fortunate to have his participation on this project. After he completes his training in both the allogeneic and syngeneic transplants, his first group will be the final cold ischemia animals, and by that time we hope to have sufficient CR2IgG isolated to start the blocking experiments.

Dr. Platt and Dr. Cascalho remain collaborators on the project, but in the rebudget for the NCE there was not sufficient remaining funds to hire the microsurgeon for year 3 and purchase the animals and supplies needed and also send funds for effort and IDC to the University of Michigan. As such they will not be receiving effort or IDC in year 3 of the grant.

With respect to the problems with immunohistochemistry, we started have very high backgrounds when staining the skin and vessels taken either as biopsies or at the endpoint. Our standard procedure was to put the tissue in formalin and then put the tissue in blocks for sectioning. The amount of time tissue was in formalin varied significantly, and we were concerned this might be the cause of the background. In the last quarter of year 2, we consulted with the pathology department at UofLHealth Jewish Hospital, which processes all of the biopsies and tissue from patients who have received organ transplants in the Trager Transplant Center, including our hand transplant recipients. We worked with one of the pathologists Dr. Kristina Wakeman who took slides from representative samples and stained them using the clinical processors for H&E, IgG and the antibody ERG, which is specific for endothelial cells. We are pleased that the staining was very good, and the H&E and ERG staining did not seem to be affected by the time in formalin. The IgG background is still higher than we would like, but Dr. Wakeman feels the samples were still interpretable. We are currently adapting the protocol and reagents from the clinical processing equipment, and Mr. Malkani will take on finishing up processing the samples for the immunohistochemistry analysis as part of his Master's research.

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

We are pleased to report that Mr. Max Malkani benefited from training or professional development during the last year. Mr. Max Malkani was an undergraduate student at the University of Kentucky that we introduced in our year 1 annual report. He wanted research experience and spent his summer break working on the project. As part of his work on this study he received animal care and laboratory safety training. Mr. Malkani became very proficient in handling the animals and performed daily monitoring and administration of tacrolimus. In addition, he learned how to mount and section tissue for immunohistochemistry, and participated in protocols for staining sections, and observed surgical procedures to transplant the osteomyocutaneous flaps. Mr. Malkani became a valued member of the research team. He submitted and presented an abstract comparing C3d and C4d staining during acute cellular rejection to Research!Louisville in 2021. After his experience, Mr. Malkani decided to pursue a Master's degree in the Department of Physiology at the University of Louisville. He submitted an abstract to Research!Louisville in 2022 and it was also accepted and presented as a poster. Mr. Malkani will be participating in this project as part of his research requirements for his Master's degree.

- **How were the results disseminated to communities of interest?**

As mentioned above, data from the project was presented at Research!Louisville and has been presented twice at the monthly Transplant Research Working Group (TRWG) meeting. In addition, we have received a review of our manuscript entitled "Mimicking Clinical Rejection in a Rat OMC Flap Model of Vascularized Composite Allotransplantation" from the Journal of Surgical Research, and were told if the requested revisions were made and accepted by the editor our manuscript would be one of three papers featured, and they also requested that we select one of our figures and send as high resolution for use on the cover.

We also presented some interesting observations on the animals transplanted to date on this study, with the expected vasculopathy generated in allogeneic recipients who received suboptimal doses of tacrolimus. What we also found was that some of our syngeneic transplants, who did not receive any tacrolimus also had vasculopathy not only on the syngeneic femoral transplant, but on the **native** femoral artery. We are in the process of analyzing the data from our syngeneic animals which also received tacrolimus, but we hypothesize that the tacrolimus may play a role in preventing this vasculopathy in the absence of alloreactivity. This abstract was presented as an oral presentation at the American Society of Reconstructive Transplantation recently held in Chicago on Nov 2-5 2022.

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

In the next quarter we plan to focus performing OMC flap transplants in the remaining cold ischemia group, and culture supernatant from the CR2Ig Hybridoma and isolate antibody from the supernatant. We will also focus on IHC of the tissue blocks we have accrued. All of the samples collected to date are now in blocks and slides have been cut in preparation for staining from most of the blocks. We initiate blockade experiments in February and complete all of the transplants by the end of July. We expect to have another manuscript in draft form during quarter 1 of year 3 which will focus on IHC and the odd vasculopathy seen in the syngeneic groups.

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?**

As part of understanding how blockade of C3d impacts the immune response to alloantigen, we assessed the binding patterns of C3d and CD21 over time in skin biopsies of animals which were

exhibiting severe ACR after cessation of tacrolimus immunosuppression, in animals with tacrolimus immunosuppression known to maintain the graft in animals with chronic rejection. These experiments have led to the previously unknown observation that in the very early post transplant period, the most intense C3d staining was found on structures within the hair follicle, but ONLY in animals that were undergoing ACR. This finding will be pursued to determine why C3d is preferentially binding there and if intervention might mitigate the ACR response. In addition, we know that the adnexal structures, including hair follicles are a target of chronic rejection in both rats and humans. This finding may also lead to an early biomarker of chronic rejection.

We have better defined our model and observed unusual graft vasculopathy in syngeneic animals which may be controlled by tacrolimus. We should be able to confirm this hypothesis in Year 3 quarter 1. We have also gained an understanding of the impact of very short (2 hours) cold ischemia time and the negative impact on graft function and survival. For reasons we are still evaluating the 2 hours of cold ischemia often resulted in excessive and sometimes uncontrolled bleeding. This issue was resolved and the animals transplanted in the groups listed in table 1 all had expected outcomes with respect to graft survival.

- **What was the impact on other disciplines?**

Nothing to report at this time.

- **What was the impact on technology transfer?**

Nothing to report at this time.

- **What was the impact on society beyond science and technology?**

Nothing to report at this time.

4. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

- **Changes in approach and reasons for change**

No changes in approach

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

The problems encountered in year 2 of the grant are described above, as were our resolutions. In brief the major impact was loss of our microsurgeon and failure of the contracted company to produce CR2Ig for blocking. We have hired a new microsurgeon and we now have hybridomas in culture and will start isolating antibody from the supernatant. In addition we have addressed issues we had with immunohistochemistry.

- **Changes that had a significant impact on expenditures**

As described above, the price of rats increased significantly, and animal loss due to surgical technique required us to use more animals than predicted. This was offset by our microsurgeon leaving, and so we used less animals during that time. In quarter 3 of year 2 we requested a NCE and

a rebudget of remaining funds to hire a microsurgeon and finish transplanting the remaining animal groups.

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

None to report

- **Significant changes in use or care of human subjects**

Not applicable

- **Significant changes in use or care of vertebrate animals.**

No significant changes

- **Significant changes in use of biohazards and/or select agents**

No significant changes

5. **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. Beare JE, Fleissig Y, Kelm NQ, Reed RM, LeBlanc AJ, Hoying JB and Kaufman CL. Mimicking Clinical Rejection in a Rat OMC Flap Model of Vascularized Composite Allotransplantation. First revision submitted and with the editor at the Journal of Surgical Research.

The pdf of this revision is attached to the report.

Other publications, conference papers, and presentations.

Poster Presentation at Research!Louisville 2022 –

Title: Focal Artery Intimal Proliferation in Absence of Alloreactivity or Surgical Trauma
. Max Malkani, Natia Q. Kelm, Jason E. Beare, Amanda LeBlanc, and Christina Kaufman

Oral Presentation at American Society of Reconstructive Transplantation, Chicago IL
Experimental Model of VCA Graft Vasculopathy and the Role of Vibrational Trauma

Jason E. Beare, Yoram Fleissig, Amanda LeBlanc, and Christina Kaufman

(Note: This presentation included data from animals in the present study as well as our previous DoD funding under W81XWH1320057)

Website(s) or other Internet site(s)

None to report

Technologies or techniques

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

None to report

○ **Other Products:**

- We have accumulated more than 700 tissue blocks from animals transplanted and have confocal and digitized light images of immunohistochemistry and H&E stained slides from ~300 tissue samples.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

○ **What individuals have worked on the project?**

Name: Christina Kaufman PhD

Project Role: PI

Nearest person month worked: 12 months at 5% effort

Contribution to Project: Dr. Kaufman is responsible for all aspects of the study, including design, conduct, data analysis, reporting and administration

Name: Amanda LeBlanc PhD

Project Role: Co-I

Nearest person month worked: 12 months at 5% effort

Contribution to Project: The studies are conducted in Dr. LeBlanc's laboratory, and Dr. LeBlanc contributes to experimental design, review of results and interpretation of data, as well as preparation of manuscripts and reporting of findings.

Name: Marilia Cacahlo MD, PhD

Project Role: Subaward Site PI

Nearest person month worked: 12 months at 5% effort

Contribution to Project: Dr. Cascalho contributes to experimental design, review of results and interpretation of data, as well as preparation of manuscripts and reporting of findings. Dr. Cascalho also provided the IgG-CR2 dimer cell line and worked with Dr. Kaufman to identify vendors and initiate bulk production of the IgG-CR2(CD21) dimer that will be used to block C3d binding in vivo. Dr. Cascalho participates in lab meetings via TEAMS.

Name: Jeffrey L. Platt MD

Project Role: Co-I

Nearest person month worked: 12 months at 5% effort

Contribution to Project: Dr. Platt contributes to experimental design, review of results and interpretation of data, as well as preparation of manuscripts and reporting of findings. Dr. Platt participates in TEAMS meetings and phone calls as well as email discussion of findings.

Name: Jason Beare

Project Role: Technician/Manager

Nearest person month worked: 12 months at 50% effort.

Contribution to Project: Mr. Beare is responsible for ordering animals and supplies and participates in animal surgeries and follow up as well as organization of IACUC submissions and training for lab members. In addition, Mr. Beare is involved in the histologic analysis of samples for this project.

Name: Natia Kelm PhD

Project Role: Microsurgeon

Nearest person month worked: 6 months at 80% effort (supported by external funding)

Contribution to Project: Dr. Kelm is supported by a training grant and is responsible for performing the microsurgery. Dr. Kelm left the project in halfway through year 2.

Name: Mr. Max Malkani

Project Role: Volunteer Undergraduate Student

Nearest person month worked: 3 months at 100% effort (Volunteer)

Contribution to Project: Mr. Malkani joined the project as a summer research volunteer. He has been a joy to work with, and quickly gained the skills needed to contribute significantly to the project including mounting and cutting tissue blocks, performing H&E staining and helping Mr. Beare with preparation for surgical procedures. Mr. Malkani is now getting his masters in Physiology and is participating in this study as part of his Master's research effort.

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

No changes

- **What other organizations were involved as partners?**

We are no longer working with Precision antibody to produce the CR2IgG.

7. SPECIAL REPORTING REQUIREMENTS

8. APPENDIX

Appendix I: A copy of the manuscript submitted to the Journal of Surgical Research has been attached.

Journal of Surgical Research

Rat Model of Vascularized Composite Allotransplantation: Mimicking Clinical Rejection Patterns

--Manuscript Draft--

Manuscript Number:	JSURGRES-D-22-01414R1
Article Type:	Regular Article
Section/Category:	Transplantation and Immunology
Keywords:	rat model; transplantation; Vascularized composite allotransplantation; rejection; Vasculopathy
Corresponding Author:	Christina Lee Kaufman, Ph.D. University of Louisville Louisville, KY UNITED STATES
First Author:	Jason E. Beare, M.S.
Order of Authors:	Jason E. Beare, M.S. Yoram Fleissig, MD Natia Q. Kelm, Ph.D., M.D. Robert M. Reed, B.S. Amanda J. LeBlanc, Ph.D. James B. Hoying, Ph.D. Christina Kaufman, Ph.D.
Abstract:	Background : Graft loss in vascularized composite allotransplantation (VCA) is more often associated with vasculopathy and chronic rejection (CR) than acute cellular rejection (ACR). We present a rat osteomyocutaneous flap model using titrated tacrolimus administration that mimics the graft rejection patterns in our clinical hand transplant program. Comparison of outcomes in these models support a role for ischemia reperfusion injury (IRI) and microvessel changes in chronic rejection of skin and large vessel vasculopathy. The potential of the surgical models for investigating mechanisms of rejection and vasculopathy in VCA and treatment interventions is presented. Materials and Methods Four rodent groups were evaluated: syngeneic controls (Group 1), allogeneic transient immunosuppression (Group 2), allogeneic suboptimal immunosuppression (Group 3), and allogeneic standard immunosuppression (Group 4). Animals were monitored for ACR, vasculopathy, and CR of the skin. Results Transient immunosuppression resulted in severe ACR within two weeks of tacrolimus discontinuation. Standard immunosuppression resulted in minimal rejection but subclinical microvessel changes, including capillary thrombosis and luminal narrowing in arterioles in the donor skin. Further reduction in tacrolimus dose led to femoral vasculopathy and CR of the skin. Surprisingly, femoral vasculopathy was also observed in the syngeneic control group. Conclusions Titration of tacrolimus in the allogeneic VCA model resulted in presentations of rejection and vasculopathy similar to those in patients and suggests vasculopathy starts at the microvessel level. This adjustable experimental model will allow the study of variables and interventions, such as external trauma or complement blockade, that may initiate or mitigate vasculopathy and CR in VCA.



November 14, 2022

Scott A. LeMaire, MD
Editor in Chief
Journal of Surgical Research

RE: Submission of Revised Manuscript to the Journal of Surgical Research

Dear Dr. LeMaire,

We are writing to request your consideration of our revised manuscript that was entitled "Rat Model of Vascularized Composite Allotransplantation: Mimicking Clinical Rejection Patterns", and is now entitled "Mimicking Clinical Rejection Patterns in a Rat Osteomyocutaneous Flap Model of Vascularized Composite Allotransplantation"—at the request of our reviewers. We have submitted a point by point response to each of the reviewer's questions, and feel their suggestions have significantly improved the manuscript.

As was requested, should the revised manuscript be acceptable for publication, we have also submitted a potential figure for the cover. We are happy to change this figure in any way needed. Of note, most of the figures with the manuscript are also submitted as high resolution if you would like to consider using one of them. Again, anything we can do to help, please let me know.

Thank you very much for the opportunity to submit our manuscript to the Journal of Surgical Research. If there is any other information we can provide, please do not hesitate to ask.

Sincerely,

A handwritten signature in black ink that reads "Christina L. Kaufman". The signature is fluid and cursive, with the first name "Christina" and last name "Kaufman" clearly legible.

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Revised 11/7/2022

Mimicking Clinical Rejection Patterns in a Rat Osteomyocutaneous Flap Model of Vascularized Composite Allotransplantation

Short Title: Rat VCA model of vasculopathy

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Authorship

A.L., J.H., and C.K. conceived and designed the research project. J.B., Y.F., R.R., and N.K. performed the surgeries and performed daily operations of the experiments. J.B., Y.F., and N.K. prepared the tissue samples, performed histological staining, and imaged them. J.B. performed histological measurements and statistical analyses of the data. J.B., Y.F., R.R., N.K., A.L., J.H., and C.K. wrote the manuscript.

Declarations of interest: none

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November 7, 2022

RE: Response to Request for Major Revision to Ms. No.: JSURGRES-D-22-01414
Title: Rat Model of Vascularized Composite Allotransplantation: Mimicking Clinical Rejection Patterns
Journal of Surgical Research

Dear Ms. Newcombe,

Please find attached a substantially revised manuscript in which we have carefully addressed the excellent comments submitted by the reviewers and editors, which we feel has significantly improved the report. As outlined below we are submitting a point by point response to each of the comments.

We are thrilled that our revised article, if accepted, may be one of the "Featured Articles" section of the journal. As you suggested, we have submitted a figure as a high resolution file that could be used on the cover of JSR. If there is any other information you need, or you would like any modifications to these figures, please let us know.

We have submitted two versions of the manuscript, a revised version of the original containing highlighted/underlined changes uploaded in the manuscript element labeled "Manuscript". In addition, we have uploaded a revised clean manuscript in the manuscript element labeled "Revision - Unmarked".

Point by Point response to Reviewers' comments:

Reviewer #1:

1. Why did the authors switch from 0.5 to 0.25 in the middle of the experiment? It would seem that having a group at 0.5 and a group at 0.25 might be a cleaner way to look at suboptimal immunosuppression. It would have been interesting to see the effect of having these distinct groups play out over time.

When we first began performing these transplant surgeries, we started with doses of tacrolimus based on the literature of 2mg/kg. This dose was effective in preventing acute rejection, but was associated with unacceptable toxicity (weight loss and failure to thrive) in our facility. After some titration experiments, we found reducing the dose of tacrolimus to 0.5mg/kg on day 12 post-transplant prevented early acute rejection and was not associated with the weight loss found with the 2mg/kg dose. We found this regimen did a good job of controlling most acute rejection and prevented the development of frank chronic rejection of the skin or frank donor artery vasculopathy. In order to allow routine development of chronic skin rejection and graft vasculopathy after approximately two weeks, we further reduced the tacrolimus dose by 50% to 0.25 mg/kg and found this dose did result in consistent development of significant chronic rejection, especially if we waited until 3 months post-transplant. We found that lower doses of tacrolimus in the first month post-transplant in our facility resulted in aggressive cellular rejection and graft loss before lesions associated with chronic rejection could develop. Thus, in our facility, the stepwise decrease in immunosuppression offered the most clinically relevant model of chronic rejection.

2. Why did ischemia/re-perfusion lead to such an effect in the syngeneic animals? Does this process also elicit an effect in the allogeneic animals? The ischemia time was only listed as 45 minutes but perhaps the limbs need to be cold perfused before surgery.

To be honest, we don't know, and this unexpected finding is under investigation. As shown in Figure 7, we did not see this vasculopathy on the recipient side of the anastomosis of the femoral artery of the allogeneic

animals. We know that patients who undergo amputation and replantation of digits and/or the upper extremity can also have graft vasculopathy, and this is attributed to trauma of the injury and subsequent surgery as well as extended ischemia. The unexpected absence of similar vasculopathy in the recipient side of allogeneic transplants suggests that ischemic time was not the only factor. On page 20 in the discussion we point out the only difference in these recipient vessels is the allogeneic animals were on tacrolimus and the syngeneic animals in our experiment (and clinical replant patients) are not on tacrolimus. We have experiments planned to determine if tacrolimus will prevent the development of this unusual vasculopathy in our syngeneic animals.

3. Is there any known difference in the rejection seen in the transient immunosuppression versus no immunosuppression. It would be interesting to look at a group of no immunosuppression versus transient immunosuppression.

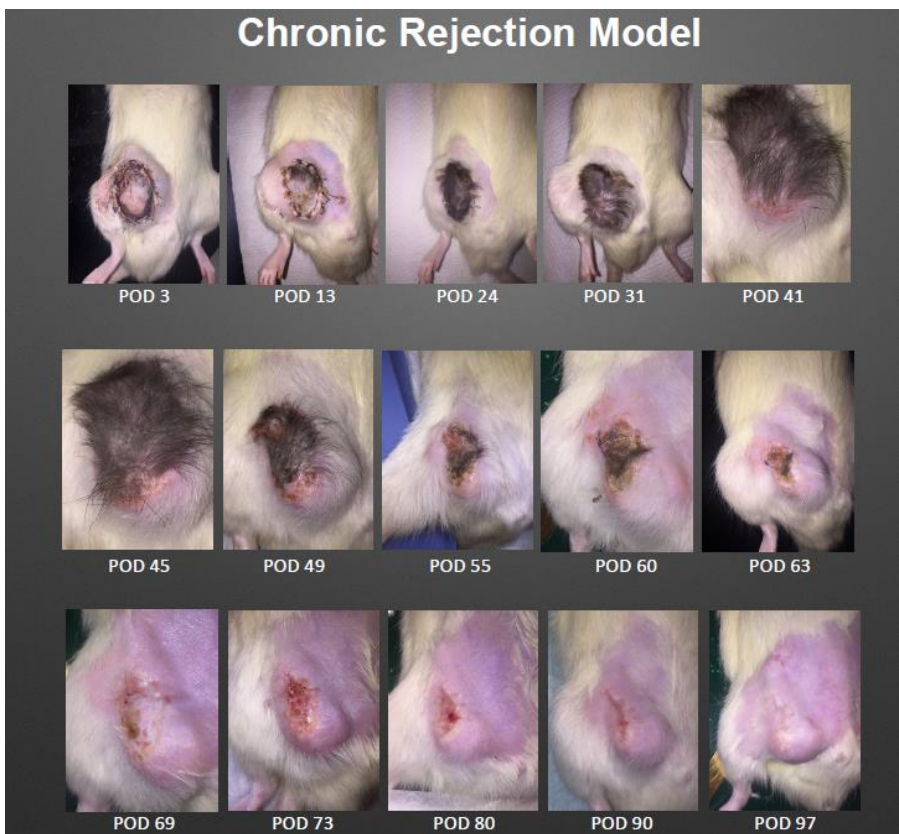
We know from previous work that in the absence of immunosuppression, Lewis rats reject Brown Norway grafts within 10-14 days. In our model of transient immunosuppression animals received 2mg/kg of tacrolimus for 11 days, which is sufficient to control rejection for that time period. There are reports in the literature stating that two weeks of tacrolimus at 2mg/kg was sufficient to prevent rejection for periods up to 122 days (Min Z and Jones NF, J Hand Surg Am 1995, 20:77) but in our facility we observed evidence of ACR by day 20 post-transplant, and frank rejection of the grafts at explant on day 60. From these observations we conclude that our transient immunosuppression model mimics severe acute cellular rejection similar to that observed in severe patient non-compliance.

4. These models appear to be primarily T cell mediated rejection (generally seen in the ACR of VCA) is there a way to administer the right doses of immunosuppression to allow for B cell and antibody mediated pathway to dominate.

The reviewer brings up an interesting idea, and one we believe could be implemented. It is possible that administration of a rat specific CD8 T cell depleting agent, allowing CD4 T cells to provide help to B cell populations, might set up the immune response for primarily BCMR to occur. A good candidate antibody for these studies might be the mouse anti-rat CD8 antibody OX-8 which has been used to deplete CD8 T cells in rats (Laberge et al J Allergy and Clinical Immunol 98:617 (1996)). If TCMR is still too strong, perhaps an initial two week course of lower tacrolimus doses might be sufficient to control that.

5. The sub-optimal immunosuppression still appears to follow a more acute rejection pattern, though possibly blunted, and exactly what is seen in the clinic. How do the author's expect this model to translate to the clinical models where chronic fibrosis and vasculopathy predominate. Are there additional strategy that could be employed to the model to allow for a more chronic pattern to emerge

In this report we focused on the development of graft vasculopathy.



However, we are pleased to share with the reviewer that our suboptimal immunosuppression model does develop gradual fibrosis of the donor skin, and severe contraction, exactly as has been observed in clinical face transplants. This fibrotic process takes longer, and is observed most often at two or more months post-transplant. As seen in the figure above (and in Figure 3D in the manuscript), at 90 days post-transplant, there is a clear demarcation of the contracted and fibrotic donor skin. Note that the muscle and bone, while affected, are much less so than the skin and vessels. Another important observation from this figure is that the epidermis that covers the graft once the fibrotic donor skin contracts has sparse, but clearly white hair, confirming the recipient Lewis origin.

Reviewer #2: Congratulations on a well planned, executed and written set of experiments elaborating on a previously published model for evaluating rejection in a rat osteomyocutaneous flap VCA model. This work is highly relevant to the future successes of VCA, both experimentally and clinically.

Objective: Develop an adaptable VCA model in rats to study acute cellular rejection and chronic rejection via titration of immunosuppression.

1. The novelty of this manuscript lay in the titrated administration of tacrolimus to mimic specific aspects of clinical graft rejection, not the VCA model itself. Consider highlighting this important contribution by reversing the title: Mimicking clinical rejection patterns in a rat model of vascularized composite allotransplantation.

We thank reviewer 2 for this excellent suggestion and have made the change in the title.

2. Page 5, donor surgery:

a. The opening sentence should indicate this manuscript utilizes an osteomyocutaneous flap model as opposed to the orthotopic models. This is only mentioned once in the abstract and is an integral component of the model.

b. Consider also mentioning the flap model in the title as well, as per the original article (ref #23).

We agree and have amended the title to include OMC flap as a descriptor, and have added OMC flap as a key word on page 5 and in both the final sentence of the introduction and the opening sentence of the description of donor surgery on page 8.

c. Only one milliliter was used to flush grafts? This volume is insufficient to flush a graft of this size. Please amend this statement accordingly.

One ml was the amount of fluid that was used to flush the grafts. The microsurgeon flushed until the effluent flowed clear, and this was documented as 1 ml for these experiments presented in this manuscript. Of note, our current procedure does use higher volumes, up to 4 ml to flush the graft after we had problems of graft failure when testing increased cold ischemia. We have included a discussion of this issue on page 9.

3. Page 6, recipient Surgery-

a. Ischemic time was 60-90 minutes. How were the limbs stored in the interim?

b. Were these surgeries staggered or concurrent?

We started accruing ischemic time when the femoral artery was cut, until the anastomosis with the recipient femoral artery was complete. In general, it took 60-90 minutes to lift the flap and perform the transplant into the waiting site recipient groin. The flap was at room temperature during the surgical procedure. The lifting of the donor flap and the preparation of the recipient site and then inset of the donor flap were concurrent with one surgeon lifting the flap and another preparing the inguinal site and inserting the donor flap. We have clarified these details in the methods section on page 9.

4. Page 17: Discussion-

Does group 3 mimic any particular patient population? The clinical comparisons for groups 2 and 4 were very

helpful for context. Is there a clinical comparison that can be made for group 3, as there are for groups 2 and 4?

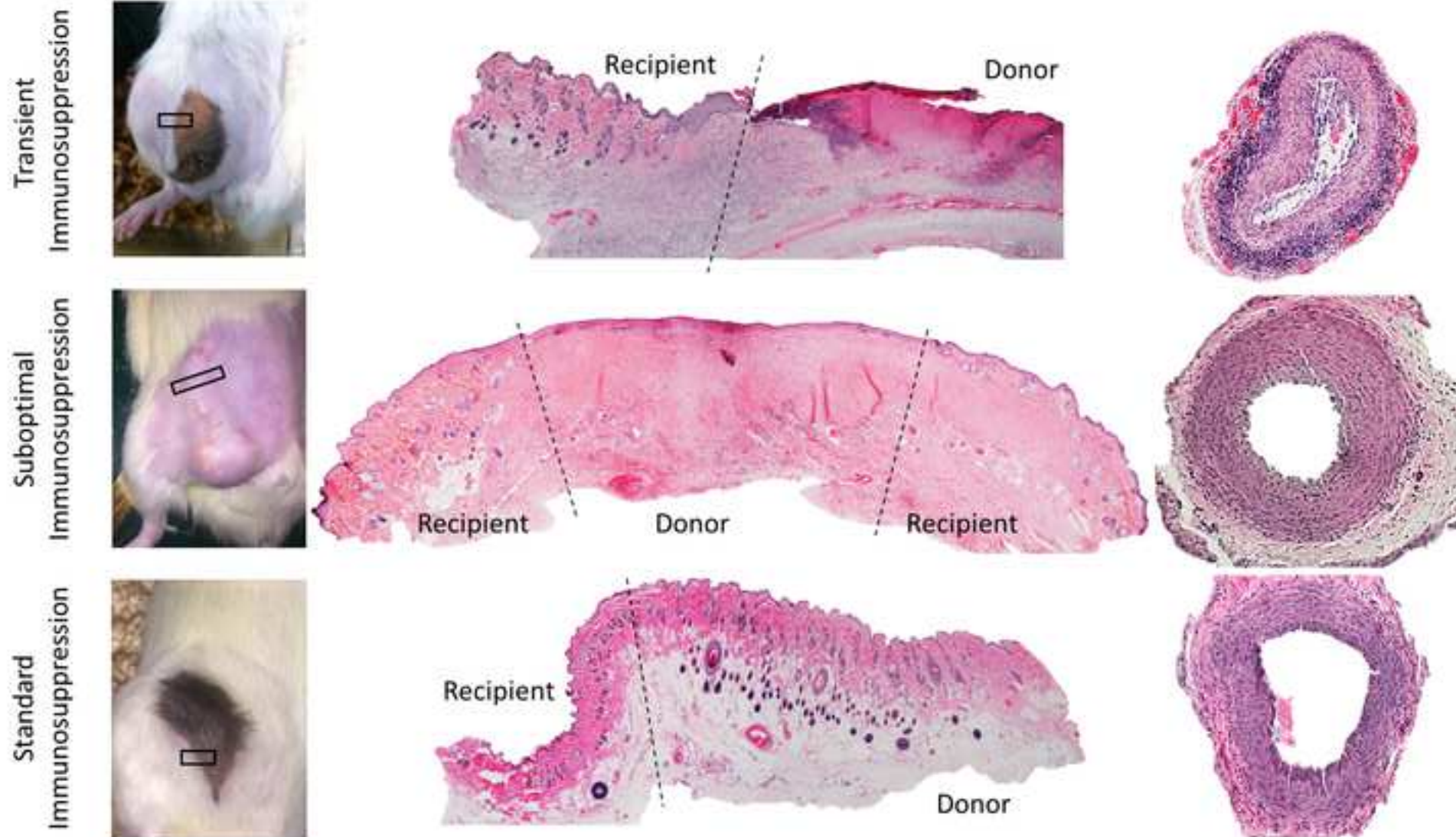
We apologize for this oversight. Group 3 is integral to this project and was designed to mimic patients with chronic rejection. As shown in figure 3D chronic rejection of the skin, with loss of adnexal structures such as hair, and contracture of the donor skin as is seen in upper extremity and face transplant recipients with chronic skin rejection. We focused on graft vasculopathy studies in this report, but the model clearly does mimic chronic rejection of both the skin and the vessels seen in clinical VCA patients in our center as well as others. We have added verbiage to page 16, 18, and 20 discussing the intent to mimic chronic rejection in group 3, as well as demonstration that these characteristics were found in Group 3 animals.

We would like to thank the reviewers and editors for their excellent questions, and feel that the manuscript is greatly improved. We hope that JSR will consider publication of the revised manuscript. We have attached high resolution versions of all figures. We have also attached a very high resolution figure which shows the three allogeneic models with donor skin and donor vessels which has nice color and may be of interest for the cover. If you would like modifications of any of these figures please let us know.

Sincerely



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This figure compares the external appearance of the osteomyocutaneous flap VCA transplant in the first column, with either transient tacrolimus immunosuppression (2mg/kg for 11 days), suboptimal immunosuppression (2mg/kg to 0.5mg/kg at day 12 and 0.25 mg/kg at day, and standard immunosuppression (2 mg/kg to day 12 and then 0.5mg/kg until the end of the study). The middle column shows the H&E histology of the strip of skin shown in the box in the first column. Both recipient and donor skin is shown. Note the necrotic donor skin in the top row, and the fibrotic scar with loss of rete ridges in the donor skin in the second row, which mimics clinical chronic rejection of the skin. In the bottom row, under standard immunosuppression, both recipient and donor skin look normal. The last column shows a section of donor femoral artery showing severe cellular rejection on the top, intimal hyperplasia and graft vasculopathy in the suboptimal IS group, and minimal vasculopathy in the standard immunosuppression group.

Revised 11/7/2022

~~Rat Model of Vascularized Composite Allotransplantation: Mimicking Clinical~~
~~Acute and Chronic Rejection~~ **Mimicking Clinical Rejection Patterns in a Rat**
Osteomyocutaneous Flap Model of Vascularized Composite Allotransplantation

Short Title: Rat VCA model of vasculopathy

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Authorship

A.L., J.H., and C.K. conceived and designed the research project. J.B., Y.F., R.R., and N.K. performed the surgeries and performed daily operations of the experiments. J.B., Y.F., and N.K. prepared the tissue samples, performed histological staining, and imaged them. J.B. performed histological measurements and statistical analyses of the data. J.B., Y.F., R.R., N.K., A.L., J.H., and C.K. wrote the manuscript.

Declarations of interest: none

Funding: This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs through the Congressionally Directed Medical Research Program under award no. W81XWH-13-2-0057 and W81XWH-20-10943, the National Institutes of Health (P30ES030283 - AJL) and the Gheen's Foundation (AJL). Opinions, interpretations, conclusions, and recommendations are those of the authors, and are not necessarily endorsed by the Department of Defense.

Abbreviations

ACR, acute cellular rejection

CR, chronic rejection

EEL, external elastic lamina

IEL, internal elastic lamina

IRI, ischemia reperfusion injury

MHC, major histocompatibility complex

POD, post-operative day

SOT, solid organ transplant

VCA, vascularized composite allotransplantation

Abstract

Background: Graft loss in vascularized composite allotransplantation (VCA) is more often associated with vasculopathy and chronic rejection (CR) than acute cellular rejection (ACR). We present a rat osteomyocutaneous flap model using titrated tacrolimus administration that mimics the graft rejection patterns in our clinical hand transplant program. Comparison of outcomes in these models support a role for ischemia reperfusion injury (IRI) and microvessel changes in chronic rejection of skin and large vessel vasculopathy. The potential of the surgical models for investigating mechanisms of rejection and vasculopathy in VCA and treatment interventions is presented.

Materials and Methods Four rodent groups were evaluated: syngeneic controls (Group 1), allogeneic transient immunosuppression (Group 2), allogeneic suboptimal immunosuppression (Group 3), and allogeneic standard immunosuppression (Group 4). Animals were monitored for ACR, vasculopathy, and CR of the skin.

Results Transient immunosuppression resulted in severe ACR within two weeks of tacrolimus discontinuation. Standard immunosuppression resulted in minimal rejection but subclinical microvessel changes, including capillary thrombosis and luminal narrowing in arterioles in the donor skin. Further reduction in tacrolimus dose led to femoral vasculopathy and CR of the skin. Surprisingly, femoral vasculopathy was also observed in the syngeneic control group.

Conclusions Titration of tacrolimus in the allogeneic VCA model resulted in presentations of rejection and vasculopathy similar to those in patients and suggests

vasculopathy starts at the microvessel level. This adjustable experimental model will allow the study of variables and interventions, such as external trauma or complement blockade, that may initiate or mitigate vasculopathy and CR in VCA.

Key Words

Rat **osteomyocutaneous flap** model, transplantation, vascularized composite allotransplantation, rejection, vasculopathy

Introduction

Clinical experience with vascularized composite allotransplantation (VCA) of the upper extremity is now in its third decade; results to date have demonstrated good outcomes with complications similar to those in solid organ transplantation (SOT(1)). In contrast to the fairly homogenous tissue composition of SOT, upper extremity and some face VCA are composed of skin, muscle, bone, and other tissues, each with its own immunogenicity characteristics. More than 80% of VCA patients exhibit acute rejection events within the first year following transplant(2, 3). This high rate of rejection has been attributed to the immunogenicity of the skin(4-6), but may also be related to the ease of diagnosing rejection in an allograft that can be visualized directly(5). Lung transplants that are directly exposed to the environment also have high rates of rejection(7), albeit not as high as those observed in VCA recipients.

Historically, graft rejection was broadly defined as acute rejection, either of a cellular or humoral nature, or chronic rejection, an indolent process that can progress to obliterative vasculopathy and parenchymal fibrosis(8, 9). The risk of acute rejection is high in the first few months post-transplant, while chronic rejection occurs months to

years post-transplant. Exposure of the recipient to allogeneic MHC on donor antigen presentation cells (APC) or direct allorecognition may result in a stronger T cell activation and response early post-transplant(10), while recipient exposure to donor MHC presented on the host APC (indirect presentation) leads to activation of a more limited T cell repertoire and graft targets, resulting in chronic rather than acute rejection processes(11). The definition of the different types of rejection is an ongoing and revolving process and is graft specific. Recently, the European Society of Organ Transplantation published an update on the current six different histological phenotypes of acute and chronic rejection in kidney transplantation(12). The definition of chronic rejection in VCA recipients is also being defined(13), and donor vasculopathy resulting in ischemic graft loss and fibrotic changes in the skin have been described(4, 14, 15). Vasculopathy in VCA is similar to that observed in cardiac transplantation, defined as diffuse stenosis caused by concentric intimal hyperplasia and inadequate compensatory outward modeling(16). Like VCA grafts(14, 17), all vessels may be involved, from the microcirculation to the large epicardial arteries and the coronary veins(18). Presentation occurs months to years after transplantation, indicating a chronic vs. acute immune response. While lesions are fairly uniform, the progression to vessel narrowing and eventually allograft ischemia(19-21) is quite focal(18). In addition to chronic rejection in the form of vasculopathy, VCA allografts have multiple CR targets, including skin and adnexal structures (hair follicles, sebaceous glands, nailbeds) as well as vessels(13).

While acute vs. chronic rejection can be somewhat defined by the temporal relation to time of transplant, acute cellular or humoral rejection can occur any time post-transplant. Acute rejection is differentiated from chronic rejection by a more

intense mononuclear cell infiltrate in parenchymal tissue and can result in significant and even necrotic tissue damage. Acute humoral rejection is also referred to as acute vascular rejection; this form of rejection is not associated with cellular infiltrates and is characterized by focal ischemia, endothelial swelling and intravascular coagulation(22). Chronic rejection of the skin is associated with minimal cellular infiltrate, with extensive fibrosis and loss of adnexal structures (i.e. hair follicles and sebaceous glands) and normal skin architecture. Multiple presentations of chronic rejection have been documented in VCA patients, including ischemic vasculopathy in the absence of ACR or skin involvement, CR of the skin, and loss of fingernails with minimal vasculopathy as well as severe ACR, vasculopathy, and chronic skin changes(13).

There remains much speculation regarding the pathogenesis of vasculopathy; factors such as inadequate immunosuppression, extensive physical activity of the hand, vibrational injury or other traumatic mechanical damage, infection, ischemia-reperfusion injury, and cell- and antibody-mediated rejection have been proposed(4, 13-15, 17, 20). The common mechanisms and presentation of acute and severe vasculopathy and CR in the skin vs. non-progressive vasculopathy between VCA recipients have yet to be elucidated.

These unanswered questions regarding the targets and presentation of CR in hand transplant and other VCA patients necessitate the development of animal VCA models that will allow for the intervention or exacerbation of ACR, vasculopathy, and skin CR during a clinically relevant course of immunosuppression. Such models will offer insights into rejection and vasculopathy in the face of interventions such as complement blockade, infectious challenge, and vibrational or other traumatic injuries.

In this study, we utilized a modified rat heterotopic **osteomyocutaneous flap** hind limb allograft(23) to develop an adaptable model of VCA with carefully titrated immunosuppression which allows for the study of ACR and CR of the skin, as well as vasculopathy, as seen in a clinical setting.

Materials and Methods

Animals

Complete major histocompatibility complex (MHC)-mismatched male Brown Norway (BN, RT1.Aⁿ) and Lewis (Lew, RT1.A^l) rats aged 12-16 weeks and weighing 275-350 grams were used as VCA donors and recipients, respectively. A cohort of Lew rats was used as syngeneic donors. Animals were obtained from Charles River Laboratories (Wilmington, MA, USA) and acclimated to the animal facility for at least one week prior to surgery. The procedures were performed in accordance with the University of Louisville Institutional Animal Care and Use Committee (IACUC protocol #18198) and NIH *Guide for the Care and Use of Laboratory Animals*(24).

Donor Surgery

Donor **osteomyocutaneous flap** grafts were recovered for implantation as previously described(23). Briefly, the femoral vessels of donor BN (allograft) or Lew (syngeneic) animals were mobilized by ligating and cutting all vessels other than those providing arterial flow and venous return from the graft, which consisted of the tibia, fibula, surrounding muscles, nerves, and skin. The knee and ankle joints were disarticulated and the artery and vein pedicles were cut proximally near the inguinal

ligament. One milliliter of heparinized saline (30 Units/ml) was injected into the artery to flush the graft. This volume was sufficient to flush all blood from the graft with clear saline effluent post-flush. Recent experience of graft failure with increased cold ischemia times have resulted in our increasing the amount of flush used to 4 ml. For the experiments presented here, 1 ml of heparinized saline was used. After graft recovery, the donor rat was euthanized by pneumothorax.

Recipient Surgery

Recipient animals were anesthetized using 2.5% isoflurane via an endotracheal tube and then prepared for graft transplantation as previously described(23). Donor vessels were anastomosed venous end-to-side, arterial end-to-end using 10-0 nylon interrupted sutures. Visual assessment of the anastomoses confirmed effective graft reperfusion. The donor and recipient surgeries were performed concurrently and the total ischemic time for the donor limb flap was 60-90 minutes during which the graft was at room temperature while anastomoses were performed. The graft was inserted into the inguinal pocket and oriented upside down, with the ankle joint superior and the knee joint inferior. Upon removal from anesthesia, the rats were placed on a heating pad for thermal support, administered meloxicam (4 mg/kg body weight) subcutaneously for pain suppression, and monitored closely until recovery. Additional meloxicam doses were administered daily until postoperative day (POD) 3.

Immunosuppressive Regimens

A schematic diagram of the four groups and dosing regimens is shown in Fig 1. Group 1 syngeneic Lewis recipients (n=3) did not receive immunosuppression throughout the experiment, emulating clinical replant recipients without alloreactivity, and were monitored daily and explanted on POD60-POD66. Allogeneic recipients in Groups 2-4 were weighed twice weekly and administered daily subcutaneous injections of 2 mg/kg tacrolimus until POD11. In the Group 2 transient immunosuppression model, which was designed to produce early aggressive ACR, immunosuppression was discontinued after POD11; animals were monitored daily until POD41-POD44, when rejection necessitated explants (n=5). The Group 3 suboptimal Immunosuppression group was designed to produce a common presentation of chronic skin changes with large vessel vasculopathy, achieved via a stepwise decrease in immunosuppression after POD11, first to 0.5 mg/kg tacrolimus (POD12-POD26) and then to 0.25 mg/kg tacrolimus from POD27 until termination at around three months or in the case of graft failure. Termination in this group occurred between POD68 and POD97 (n=5). Finally, the Group 4 standard immunosuppression group was modeled on a clinical dose of tacrolimus known to maintain grafts with minimal ACR; tacrolimus was reduced after POD11 to 0.5 mg/kg until termination on POD60-POD64 (n=4).

VCA Recipient Monitoring

Recipient rats were housed singly and monitored daily for clinical signs of pain, dehydration, weight loss, and decreased activity, in addition to surgical failure (first 48-72h) or rejection. The initial clinical signs of rejection, including edema, maculopapular rash, erythema, and skin desquamation(25, 26) were recorded. Macroscopic images of

the grafted area were captured daily and scored for rejection by a double-blinded observer.

Laser Doppler Imaging

A Moor LD12-IR laser Doppler scanner (Moor Instruments, Wilmington, DE, USA) with an infrared 785 nm laser diode was used to assess blood perfusion through the graft skin on POD14 and the day of explant. Perfusion through the skin was measured on a colorimetric scale from no flow (blue) to high flow (red) (Fig. 2).

Tissue Biopsy and Explant Surgery

On POD14 and biweekly thereafter, recipient rats were anesthetized using 2.5% inhaled isoflurane, 2 mm skin biopsies were obtained, and the wound was closed with absorbable 4-0 suture. For explant, animals were deeply anesthetized with 5% inhaled isoflurane; larger biopsies of the donor-recipient skin interface were procured, as well as donor muscle biopsy. Arterial samples (1-2 mm in length) were obtained proximally (recipient side) and distally (donor side) to the anastomosis (Fig. 4A). All tissue samples were fixed in 10% formalin before paraffin embedding.

Histologic Evaluation

For morphometry, 6 μ m sections of skin, muscle, and vessel samples were stained with hematoxylin and eosin (H&E) using standard staining methods. Skin biopsies were examined for changes in skin morphology and architecture as well as

loss of adnexal structures (i.e., hair follicles and sebaceous glands). Skin and muscle biopsies were graded by a blinded observer, using the criteria listed in Table 1.

Evaluation of Graft Vasculopathy

All measurements were performed by an observer blinded to the sample location and immunosuppression group. The vessel lumen area as well as the internal and external elastic laminae (EEL) were traced using ImageJ analysis software (NIH, Bethesda, MD), allowing for the calculation of intimal and medial thickness and area. The total number of cell nuclei within the medial layer of each vessel was determined. Percent intimal proliferation was calculated as intimal thickness/total laminae thickness, and the occlusion ratio was calculated as lumen diameter/total vessel diameter. For skin microvessel analysis, the total number of microvessels (defined as vessels with a lumen diameter of less than 200 μm) was determined for the donor skin area as well as the recipient skin area. The percentage of microvessels occluded by thrombosis, lumen narrowing, and/or microvessel wall thickening was calculated separately for donor and recipient skin areas.

Statistical Analyses

Statistical analyses were performed using SigmaPlot 14.0 (Systat, San Jose, California, USA) with $p < .05$ significance level. Within group and within location (donor, recipient, and contralateral artery) one-way ANOVA was performed on all histological vessel measurements, followed by Holm-Sidak *post hoc* analyses where appropriate.

To ensure rigor, histological images were coded and analyzed in a blind fashion with the code being broken only after all scoring quantifications were acquired.

Results

Operative results

The donor operative time was approximately 45 min, while the recipient operative time was approximately 1.5 to 2 h. On rare occasions, minor postoperative complications, such as focal skin necrosis, usually on the caudal edge of the skin paddle, and iatrogenic skin necrosis following subcutaneous tacrolimus injections, were observed. Upon explant analysis, one animal in the standard immunosuppression group was found to have numerous masses in the inguinal cavity, peritoneal cavity, and lungs; this animal was excluded from histological analyses.

Graft Perfusion

Laser Doppler imaging of syngeneic animals revealed proper blood flow through the skin of the graft throughout the study. Macroscopic examination of the femoral artery revealed no signs of trauma or hypertrophy on either side of the anastomotic site (data not shown).

Group 2 transient immunosuppression animals exhibited sufficient perfusion to the graft on POD14, including robust recipient collateral feeding vessels (Fig. 2A). Blood perfusion to the skin was completely ablated on the day of explant (POD43)(Fig. 2B). Surgical exposure of the anastomoses during the explant revealed a healthy recipient

artery, whereas the donor artery distal to the anastomosis was severely inflamed and hypertrophied (Fig. 2C).

On POD14, Group 3 suboptimal immunosuppression animals presented with proper blood perfusion throughout the graft and abundant recipient collateral vessels (Fig. 2D), and terminal assessment revealed some areas of diminished blood flow within the VCA (Fig. 2E). Despite some loss of flow, macroscopic examination of the anastomoses did not indicate any obvious signs of vascular injury distal to the anastomosis (Fig. 2F).

In Group 4 standard immunosuppression animals, blood perfusion to the skin remained unchanged from POD14 (Fig. 2G) to POD64 (Fig. 2H), indicating sufficient blood flow throughout the course of the study. Exposure of the anastomoses at the explant revealed no outward signs of vascular trauma or thrombosis (Fig. 2I).

Clinical Signs of Rejection and Skin Histopathology

The Group 1 syngeneic control recipients exhibited normal healing, healthy skin components, and proper donor hair growth throughout the study. No clinical signs of rejection were noted. Biopsies from POD14 as well as at the time of explant (POD60-POD66) showed normal skin adnexal components. Interestingly, all the skin biopsies from Group 1 animals at two weeks showed grade1-2 cellular infiltrate (average histology score of 1.33, Table 1), with resolution of infiltrate by the time of explant (average skin histology score of 0.33, Table 1), possibly in response to IRI and surgical trauma.

In Group 2 transient immunosuppression animals, the skin showed normal wound healing and donor hair growth during the first 3 weeks post-surgery, indicating proper vascularization of the flap. The identification of donor skin vs. recipient skin was readily apparent because of the color and growth direction of the hair. The first signs of acute rejection, including skin redness, swelling, and partial loss of hair, were observed on $\text{POD}19.4 \pm 2.87$ days (Table 2); by the time of explant, severe acute rejection of donor skin and graft failure was evident in all animals (Fig. 3A). Skin histopathology from POD14 revealed normal morphology with healthy adnexa, hair follicles, and sebaceous glands; mild mononuclear cell infiltration was apparent (Grade 1-2 ACR score). In the explant, the donor skin lacked adnexal structures, and the epidermis was necrotic and atrophic (Fig. 3B). Microvessel thrombosis (Fig. 3C, white arrow) and extensive mononuclear cell infiltration were observed in the donor skin.

In Group 3 animals receiving suboptimal immunosuppression, clinical follow-up showed a normal VCA skin appearance with healthy hair growth on POD14. The identification of donor vs. recipient skin was readily apparent. Skin biopsies on POD14 showed normal morphology with diffuse mononuclear cell infiltration (Grade 2 ACR score, Table 1). When immunosuppression therapy was decreased to 0.25 mg/kg, rejection events including maculopapular lesions, gradual hair loss, and atrophy of donor muscle and skin were noted; the first rejection event was observed at $\text{POD}39.8 \pm 3.57$ days (Table 2). The donor skin over the flap gradually reduced in size over the course of the experiment and was eventually covered with a new epidermis (Fig. 3D). In explants, mononuclear cell infiltration was apparent in the donor skin (Fig. 3E), but to a lesser extent than that observed in ACR animals. Histopathology revealed a lack of

adnexal structures in the donor skin, with severely contracted fibrotic donor skin with loss of the rete ridges and epidermal thinning. → New skin likely of recipient origin (hair growth in this area was white in color suggesting Lewis strain origin) covered the rest of the muscle and bone graft. The recipient skin exhibited healthy adnexal structure, epidermis, and vessel components. At greater magnification, arteriolar wall thickening with perivascular infiltration (Fig. 3F, black arrow) was observed in the donor area of the skin.

In the Group 4 standard immunosuppression group, clinical follow-up revealed normal VCA skin and hair throughout the study (Fig. 3G). When immunosuppression was maintained at 0.5 mg/kg, the recipient animals did not exhibit gross signs of rejection. One animal in this group showed redness and swelling on POD44. However, this animal developed tumors throughout the graft and in the peritoneal cavity and lungs, necessitating its removal from the study. Skin biopsies on POD14 showed no signs of adnexal structure loss with minimal mononuclear cell infiltration (primarily ACR Grade 1), similar to both rejection groups (Table 1). Skin biopsy of the recipient/donor graft border at the explant revealed that the donor skin resembled the recipient skin. The skin exhibited normal epidermis and adnexa without morphological change, and mild mononuclear infiltration (Fig. 3H). At a higher magnification, occasional microvascular thrombosis (white arrow) and vessel wall thickening (black arrow) were observed in the donor skin (Fig. 3I).

Vessel Histopathology and Quantitative Measurement

Samples of the femoral artery proximal (recipient) and distal (donor) to the anastomosis were obtained (Fig. 4A). Histological analyses of the Group 1 syngeneic donor, recipient, and contralateral artery samples revealed no differences in either the total number of cells (Fig. 6A) or cells/ μm^2 (Fig. 6B) in the intimal and medial laminae of the vessel. The donor lumen area was slightly, but not significantly, constricted (Fig. 6C). A layer of apoptotic cells lined the lumen along the intimal interface on both the recipient and donor sides of the anastomosis. No differences were observed in intimal thickness (Fig. 6D), medial thickness (Fig. 6E), total lamina thickness (Fig. 6F), intimal proliferation (Fig. 7A), occlusion ratio (Fig. 7B), percentage of occluded skin microvessels (Fig. 7C), or skin microvessels/ mm^2 (Fig. 7D).

In the Group 2 transient immunosuppression animals, designed to develop severe acute rejection of the skin, the femoral artery exhibited changes consistent with necrotic rejection rather than vasculopathy. A robust mononuclear cell invasion in the medial layer of the donor artery (Fig. 4E) was detected compared to that in the recipient artery (Fig. 4B). An apoptotic layer of cells lining the lumen of the distal artery sample was observed (Fig. 4E), with pronounced microvascular thrombosis in the adventitial layer (Fig. 4E; examples shown with black arrows). Severe vasculitis of the donor artery in this group required imaging of the vessel sample at a lower magnification (10x); all other vessel images in Fig. 4 were acquired at 20x magnification. Quantitative vessel measurements did not show significant differences in total cells (Fig. 6A) and cells/ μm^2 (Fig. 6B) in the intimal+medial laminae. The lumen area (Fig. 6C) and intimal thickness (Fig. 6D) were unchanged, with increased medial thickness in the donor artery distal to the anastomosis compared to contralateral vessel samples (one-way ANOVA, DF=2,

F=8.081, $p=.006$, Holm-Sidak *post hoc* $p=.005$)(Fig. 5E). Similarly, the total laminae thickness (intimal+medial layers) exhibited significant outward remodeling in the donor artery compared to the contralateral control vessel (one-way ANOVA, DF=2, F=6.090, $p=.015$, Holm-Sidak *post hoc* $p=.016$. Intimal proliferation and occlusion ratios were unchanged between donor, recipient, and contralateral vessel samples (Fig. 7A and 7B, respectively). Significantly more donor skin capillaries were occluded compared to syngeneic donor skin (one-way ANOVA, DF=3, F=9.785, $p=.001$, Holm-Sidak *post hoc* $p=.001$)(Fig. 7C).

Group 3 was designed to replicate chronic rejection patterns seen in clinical VCA recipients, including chronic skin rejection with fibrosis and loss of adnexal structures and rete ridges as well as intimal hyperplasia and medial hypertrophy or graft vasculopathy in the donor vessels. In Group 3 suboptimal immunosuppressed animals, vessel samples from the recipient side of the femoral artery anastomosis exhibited healthy intimal and medial layers (Fig. 4C). Marked intimal hyperplasia was apparent in the donor artery (Fig. 4F, yellow bracket) and apoptotic cells lined the innermost layer of the intima along the lumen. Significantly more infiltrating cells/ μm^2 of the intimal+medial area was found (one-way ANOVA, DF=2, F=6.092, $p=.015$) both in the recipient ($p=.023$) and donor arteries ($p=.031$) than in the contralateral arteries (Fig. 6B). The lumen area was unchanged (Fig. 6C), while the intima was significantly thicker in the donor vessel than in the contralateral control artery (one-way ANOVA, DF=2, F=5.747, $p=.018$, Holm-Sidak *post hoc* $p=.016$)(Fig. 6D). The medial thickness and total lamina thickness remained unchanged (Fig. 6E and 6F, respectively). Intimal proliferation was significantly higher in donor vessels (one-way ANOVA, DF=2, F=13.310, $p<.001$) than in

both recipient ($p=.005$) and contralateral arteries ($p=.001$)(Fig. 7A). The occlusion ratio was unchanged (Fig. 7B), while the percentage of occluded skin microvessels was significantly higher in the donor skin than in Group 1 syngeneic donor skin (one-way ANOVA, $DF=3$, $F=9.785$, $p=.001$, Holm-Sidak *post hoc* $p=.004$)(Fig. 7C). Skin microvessels/mm² remained unchanged (Fig. 7D).

Groups 2-4 all exhibited normal vessel components on the recipient side of the anastomosis (Fig. 4B,4C and 4D). Unexpectedly, 2/3 animals in Group 1 showed mild intimal hyperplasia on the recipient side of the anastomosis (Fig. 5C, 5D). In addition, the syngeneic control animals showed moderate to severe intimal hyperplasia in 2/3 animals on the donor side of the anastomosis (Fig. 5E, 5F). Evidence of intimal hyperplasia was observed in the donor vessel of Group 4 (Fig. 4G), with a layer of apoptotic cells lining the innermost layer of the intima, but not to the extent seen in Group 3 animals who showed overt intimal hyperplasia in the donor vessel (Fig 4F). Group 2 animals who received transient immunosuppression that resulted in severe necrotic acute rejection had donor arteries with extensive lymphocytic infiltration and capillary thrombosis of the adventitial layer, and acentric intimal and medial proliferation consistent with severely inflamed tissue (Fig. 4E).

In addition to skin histology, we also scored biopsies of muscle taken at time of explant. Muscle showed cellular infiltrate and evidence of tissue damage in a similar pattern as seen for skin biopsies taken at explant. Biopsies were scored as defined in Table 1, and showed mild to diffuse cellular infiltrate in Group 1 syngeneic animals, and Group 4 standard Immunosuppression. As expected, muscle at explant from animals with severe ACR (Group 2) revealed robust cellular infiltration, lack of cross striation

and hypochromagenicity of the muscle, and arteriole hyperplasia (black arrow, Fig. 8A) (Table1). Muscle biopsy from Group 3 Suboptimal Immunosuppression animals revealed moderate cellular infiltration and decreased lumen diameter in the muscle arterioles (Fig. 8B, black arrows) (Table 1). In contrast, Histological analysis of the explant muscle samples from Group 4 standard immunosuppression animals revealed mild cell infiltration and healthy microvessels (Fig. 8C, white arrow) (Table 1).

Discussion

Multiple rodent experimental models of VCA have been described, each with its own advantages and drawbacks(27). The model presented here was developed at our center(23) and offers key components that mimic various presentations of rejection observed in the clinic. The groups presented were successful in modeling patterns seen in clinical replant patients (Group 1 – Syngeneic transplants in the absence of immunosuppression), and in clinical VCA transplant recipients including ACR without vasculopathy (Group 2 – Transient immunosuppression), evidence of both chronic skin changes and vasculopathy (Group 3 – Suboptimal immunosuppression), and minimal ACR with some microvessel thickening in the absence of significant large vessel involvement or significant acute chronic skin rejection (Group 4 – Standard immunosuppression).

The rejection spectrum demonstrated by the groups in this study is influenced by four variables: IRI/surgical trauma, allogeneic donor MHC, the level of immunosuppression and duration of immunosuppression. Group 1 animals showed an unexpected amount of intimal proliferation on both the recipient and donor side of the

anastomosis. Of relevance to the observation in this report of vasculopathy in the donor vessel of our syngeneic control groups, vasculopathy can also occur when digits and upper extremities are replanted following amputation(28, 29). This vasculopathy is not related to alloantigen and likely originates in the ischemia reperfusion injury (IRI) to the replanted tissue as a result of the trauma and surgical procedure.

A characteristic shared by Group 1 and clinical replant patients is that neither received tacrolimus immunosuppression. As we did not see significant hyperplasia on the recipient side of the anastomosis in Group 2-4, nor in the donor femoral artery in Group 2 animals, it may be that the tacrolimus interfered with the development of non-allogeneic related intimal hyperplasia. A future direction of ours is to repeat Group 1 and also test the ability of tacrolimus to interfere with the development of intimal hyperplasia due to IRI and surgical trauma. In the Group 2 transient immunosuppression group, when immunosuppression was halted 12 days postoperatively, severe acute rejection events occurred in less than two weeks, resulting in necrotic compromise of the skin and ischemic changes before vasculopathy could develop. This model mimics the rare non-compliance situation where patients stop taking immunosuppression. In the Group 3 suboptimal immunosuppression group, tacrolimus administration was extended but reduced in a stepwise fashion and maintained at lower doses until terminal time points, which allowed both chronic skin rejection with loss of adnexal structures and moderate to severe vasculopathy to develop. In the Group 4 standard immunosuppression animals, the tacrolimus dose was lowered on POD12 but maintained at a dose sufficient to keep the graft outwardly healthy. On the surface, the skin and hair appeared normal throughout the course of the study and blood perfusion was uniform throughout the flap

at the explant. This group models patients on standard immunosuppression that are doing well clinically, but over time develop histologic changes in skin biopsies and mild intimal hyperplasia of donor vessels. In Group 4 animals, skin and vessel histology at explant showed scattered microvessel narrowing and thrombosis and mild medial hyperplasia of the donor femoral artery distal to the anastomosis. We expect mild intimal hyperplasia would also develop if animals were maintained longer. This presentation is most similar to the clinic, where the target is the lowest amount of immunosuppression that will maintain the graft, while minimizing immunosuppression complications. While Group 4 models most transplant recipients with good graft function but evidence of ongoing low-level rejection in the skin and vessels, it does not model the confluent aggressive ischemic vasculopathy and graft loss in the absence of skin rejection seen in our fourth transplant recipient(20). We hypothesize that this type of rejection is a combination of alloreactivity and external physical trauma to the graft. We have evidence that vibrational trauma can exacerbate vasculopathy in this rat model of VCA (unpublished data), and other groups have demonstrated the potential role of physical and thermal trauma in promoting graft rejection in VCA recipients(30, 31).

These models will be used in experiments to test potential interventions or define mechanisms that result in graft rejection. VCA patients that are doing well clinically but have histologic evidence of alloimmune-mediated damage are vulnerable to progression from a number of causes. Reduced doses of immunosuppression occur clinically because of related complications, neoplasia, or compliance issues. In addition, we expect accumulation of cellular- and antibody-mediated rejection events, infection,

and/or physical trauma will promote progression to chronic rejection of the skin and vasculopathy in our animals.

In the Group 4 standard immunosuppression model, it appears the vascular injury in the graft begins in the small vessels. Microvessels in the donor skin exhibited thrombosis and lumen narrowing, with minimal signs of vasculopathy in the donor femoral artery. This observation may indicate a cascade of vascular involvement in rejection that begins in the smallest vessels. Strengthening this argument, Kanitakis et al. have observed capillary thrombosis prior to vasculopathy that ultimately led to the loss of two grafted fingers in a VCA patient(32). While multiple mechanisms have been proposed for developing vasculopathy, few studies have focused on the initiating events, and provide evidence that the microvessels are affected first, as seen in Group 4.

It is important to consider the limitations and opportunities for development of the current study. While the analysis of acute vs. chronic rejection of the skin was based on mononuclear cell infiltrate and patterns of damage and fibrosis by H&E, as is done clinically, we performed staining of CD3, CD45RA and several other lineage markers which produced expected staining patterns. However, the background staining was unexpectedly high, despite multiple attempts to optimize with different blocking agents. We suspect that fixation in 10% formalin for extended periods may have contributed to the high backgrounds and this data could not be included. Additionally, two other clinical presentations of rejection seen on our hand transplant recipients remain to be developed. First is a model of development of chronic skin changes in the absence of significant vasculopathy, which has also been observed in VCA patients(13).

Additionally, we are working on a model that mimics aggressive confluent ischemic vasculopathy without ACR in the skin that resulted in early graft loss in our fourth hand transplant recipient(20).

Conclusions

Here, we present a reliable and reproducible tool for studying the underlying mechanisms of VCA graft vasculopathy and acute and chronic rejection of the skin. Careful titration of immunosuppression in this model can push a graft towards different levels of rejection, allowing for later administration of additional graft stressors (i.e. infection, mechanical insult, etc.) or interventions such as complement blockade to ascertain potential thresholds that could ultimately identify mechanisms and potential for prevention or treatment of skin CR and vasculopathy.

Figure Legends

Figure 1. Schematic diagram of the tacrolimus dosing regimens utilized to in control (Group 1) and experimental groups (Groups 2-4).

Figure 2. Laser Doppler imaging of a representative animal from each experimental group, with the border of the skin window marked by white dashed lines. Using transient immunosuppression (Group 2), robust perfusion through the graft as well as recipient collateral superficial blood supply was observed at POD14 (A) followed by complete loss of blood flow at POD43 (B). Exposure of the anastomosis (C, white arrow) at time of explant revealed severe vascular inflammation and edema distal to the anastomosis (C, black arrow). With suboptimal immunosuppression (Group 3), blood flow to the graft was normal at POD14 (D); by explant at POD90, nearly half of the graft exhibited loss of blood flow (E). The femoral artery appeared normal on both sides of the anastomosis (F, white arrow). With standard immunosuppression (Group 4), blood perfusion was normal throughout the entirety of the graft both at POD14 (G) and at explant on POD64 (H). The femoral artery exhibited normal morphology near the anastomosis site (I, white arrow).

Figure 3. Skin histopathology. At time of explant, Group 2 transient immunosuppression animals exhibited necrotic skin over the graft (A). Skin samples taken at the donor-recipient skin border (A, black box) showed massive mononuclear cell infiltration on both sides of the border, and necrotic epidermis as well as loss of skin adnexal

components on the donor side (B); further magnification of donor skin (B, black box) showed perivascular infiltration as well as microvascular thrombosis (C, white arrow). Group 3 suboptimal immunosuppression animals exhibited pervasive hair loss and contraction of donor skin consistent with chronic rejection of the skin (D). A skin sample traversing donor-recipient-donor skin border revealed moderate mononuclear cell infiltrate localized to the donor tissue (E). A healthy new layer of epidermis was observed over the donor skin component of the graft; further magnification of donor skin (E, black box) revealed perivascular infiltration and narrowing of the arteriole lumen (F, black arrow). Animals in the standard immunosuppression group exhibited normal skin and hair growth throughout the entirety of the study (G). A skin sample taken at the donor-recipient skin border (G, black box) revealed normal skin on both sides of the graft, including healthy epidermis and normal skin adnexal components (H). Further magnification (H, black box) indicated vessel wall thickening of a microvessel (I, black arrow) and microvascular thrombosis (I, white arrow). Scale bar for B, E, and H = 500 μm ; scale bar for C, F, and I = 100 μm .

Figure 4. Femoral artery histopathology. Samples of the recipient femoral artery and donor femoral artery were obtained proximal and distal to the anastomosis site, respectively (A). In groups 2-4, recipient samples exhibited a thin intimal layer (yellow arrows) and comparable medial (red brackets) and adventitial layers (blue brackets)(B-D). Acute Rejection animals showed massive mononuclear cell infiltration in both the media (red bracket) and adventitia (blue bracket), as well as microvascular thrombosis of the *vaso vasorum* (black arrows show several examples), but no significant intimal

hyperplasia (E). Suboptimal immunosuppression animals exhibited marked intimal hyperplasia (yellow bracket) with a layer of apoptotic endothelial cells lining the lumen, but relatively unchanged media (red bracket) and adventitia (blue bracket)(F). Group 4 standard immunosuppression animals exhibited signs of apoptosis of the endothelial lining and intima (yellow bracket), with slight outward remodeling of the media (red bracket) and unchanged adventitia (blue bracket)(G). Scale bar = 100 μ m for all panels; images were captured at 20x magnification for all panels except E, which was captured at 10x magnification.

Figure 5. Femoral artery histopathology of Group 1 syngeneic control animals. Samples of the recipient femoral artery and donor femoral artery were obtained proximal and distal to the anastomosis site, respectively (A). Unlike Groups 2-4, two of the three animals in Group 1 showed some level of intimal hyperplasia on the recipient side of the anastomosis. (C,D). In addition, microvascular thrombosis of the *vaso vasorum* (black arrows show several examples) were seen in the adventitia of the recipient side sample in the third animal (D). When the donor side of the femoral artery was examined, again, two of the three animals showed significant intimal proliferation, even more than seen on the recipient side of the anastomosis (E,F). The third animal showed no intimal proliferation and normal histology of the donor femoral artery (G). Scale bar = 100 μ m for all panels; images were captured at 20x magnification for all panels.

Figure 6. Quantification of total nuclei in the intimal+medial laminae (A), nuclei/ μm^2 in intimal+medial laminae (B), lumen area (C), intimal thickness (D), medial thickness (E), and total laminae thickness (F) from femoral artery histological samples. All graphs display group means + standard error. * $p < .05$ when compared to contralateral artery sample from the same group. Transient immunosuppression was characterized by cell infiltration to the donor vessel, increased medial thickening and increased total laminae area due to outward remodeling, whereas suboptimal immunosuppression resulted in cell infiltration to both donor and recipient vessel as well as intimal hyperplasia. Standard immunosuppression resulted in no large vessel differences compared to the animals' unoperated control vessel or syngeneic surgical controls.

Figure 7. Quantification of intimal proliferation (A), occlusion ratio (B), and percentage of skin microvessels occluded (C). All graphs display group means + standard error. * $p < .05$ when compared to contralateral vessels from the same group, # $p < .05$ when compared to syngeneic samples, ^ $p < .05$ when compared to recipient vessel samples from the same group. All groups exhibited significantly higher percentage of occluded microvessels in donor skin compared to syngeneic controls.

Figure 8. Representative images of muscle biopsy at the time of explant collection for each group. Animals in the transient immunosuppression (Group 2) exhibited robust mononuclear cell infiltration as well as hyperplasia of capillary vascular structures (A, black arrow). Group 3 suboptimal immunosuppressed animals showed moderate cell

infiltration, combined with compromised lumen diameter in muscle capillaries (B, black arrows). Group 4 standard immunosuppression resulted in minimal cell infiltration, healthy muscles, and normal capillaries (C, white arrow). Scale bar = 100 μ m.

Table 1. Rejection scoring for skin and muscle biopsies. All samples taken from Allogeneic transplants (Groups 2-4) at POD14 exhibited mild cell infiltration. Surprisingly Group 1 syngeneic recipients showed mild infiltrate at two weeks, which resolved by the time of explant. At explant, severe ACR with major epidermal necrosis was seen in Group 2, moderate infiltrate with some epidermal loss in Group 3 suboptimal immunosuppression animals, and mild to moderate infiltrate in Group 4 standard immunosuppression animals. Muscle biopsies at explant showed similar patterns, with mild cell infiltrate in Group1 syngeneic animals, heavy mononuclear cell infiltration with myocyte necrosis in Group 2 transient Immunosuppression animals, moderate cell infiltrate in Group 3 animals, and mild/diffuse cell infiltrate with minimal tissue damage in Group 4 standard immunosuppression animals.

Table 2. Gross clinical observation of graft. A t-test on time to first rejection event revealed significantly delayed rejection events in Group 3 suboptimal Immunosuppression animals compared to Group 2 transient immunosuppression animals (*p=.002).

References

1. Kaufman CL, Bhutiani N, Ramirez A, Tien HY, Palazzo MD, et al. Current Status of Vascularized Composite Allotransplantation. *Am Surg* 2019;85:631-637.
2. Petruzzo P, Lanzetta M, Dubernard JM, Landin L, Cavadas P, et al. The International Registry on Hand and Composite Tissue Transplantation. *Transplantation* 2010;90:1590-1594.
3. Kueckelhaus M, Fischer S, Seyda M, Bueno EM, Aycart MA, et al. Vascularized Composite Allotransplantation: Current standards and novel approaches to prevent acute rejection and chronic allograft deterioration. *Transpl Int* 2016;29:655-662.
4. Kollar B, Kamat P, Klein HJ, Waldner M, Schweizer R, et al. The Significance of Vascular Alterations in Acute and Chronic Rejection for Vascularized Composite Allotransplantation. *J Vasc Res* 2019;56:163-180.
5. Kaufman CL, Marvin MR, Chilton PM, Hoying JB, Williams SK, et al. Immunobiology in VCA. *Transpl. Int* 2016;29:644-654.
6. Kadono K, Gruszynski M, Azari K, Kupiec-Weglinski JW Vascularized composite allotransplantation versus solid organ transplantation: innate-adaptive immune interphase. *Curr Opin Organ Transplant* 2019;24:714-720.
7. Costa J, Benvenuto LJ, Sonett JR Long-term outcomes and management of lung transplant recipients. *Best Pract Res Clin Anaesthesiol* 2017;31:285-297.
8. Libby P, Pober JS Chronic rejection. *Immunity* 2001;14:387-397.
9. Demetris AJ, Murase N, Lee RG, Randhawa P, Zeevi A, et al. Chronic rejection. A general overview of histopathology and pathophysiology with emphasis on liver, heart and intestinal allografts. *Ann Transplant* 1997;2:27-44.
10. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, et al. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* 1997;64:795-800.
11. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, et al. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* 2001;167:7199-7206.
12. Becker JU, Seron D, Rabant M, Roufosse C, Naesens M Evolution of the Definition of Rejection in Kidney Transplantation and Its Use as an Endpoint in Clinical Trials. *Transpl Int* 2022;35:10141.
13. Kaufman CL, Kanitakis J, Weissenbacher A, Brandacher G, Mehra MR, et al. Defining chronic rejection in vascularized composite allotransplantation-The American Society of Reconstructive Transplantation and International Society of Vascularized Composite Allotransplantation chronic rejection working group: 2018 American Society of Reconstructive Transplantation meeting report and white paper Research goals in defining chronic rejection in vascularized composite allotransplantation. *SAGE Open Med* 2020;8:2050312120940421-2050312120940421.

14. Morelon E, Petruzzo P, Kanitakis J Chronic rejection in vascularized composite allotransplantation. *Curr Opin Organ Transplant* 2018;23:582-591.
15. Krezdorn N, Lian CG, Wells M, Wo L, Tasigiorgos S, et al. Chronic rejection of human face allografts. *Am J Transplant* 2019;19:1168-1177.
16. Mitchell RN, Libby P Vascular remodeling in transplant vasculopathy. *Circ. Res* 2007;100:967-978.
17. Kanitakis J, Petruzzo P, Badet L, Gazarian A, Thaunat O, et al. Chronic Rejection in Human Vascularized Composite Allotransplantation (Hand and Face Recipients): An Update. *Transplantation* 2016;100:2053-2061.
18. Laks JA, Dipchand AI Cardiac allograft vasculopathy: A review. *Pediatr Transplant* 2022;26:e14218.
19. Diefenbeck M, Nerlich A, Schneeberger S, Wagner F, Hofmann GO Allograft vasculopathy after allogeneic vascularized knee transplantation. *Transpl. Int* 2011;24:e1-e5.
20. Kaufman CL, R O, BW B, JE K, Tsai TM, et al. Graft Vasculopathy in Clinical Hand Transplantation. *Am. J Transplant* 2012;12:1004-1016.
21. Shetty M, Chowdhury YS Heart Transplantation Allograft Vasculopathy. *StatPearls. Treasure Island (FL):* 2021.
22. Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, et al. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999;10:2208-2214.
23. Fleissig Y, Reed RM, Beare JE, LeBlanc AJ, Williams SK, et al. Modified Heterotopic Hindlimb Osteomyocutaneous Flap Model in the Rat for Translational Vascularized Composite Allotransplantation Research. *J Vis Exp* 2019.
24. , Guide for the Care and Use of Laboratory Animals, 9th edition. Washington (DC): 2011.
25. Sarhane KA, Tuffaha SH, Broyles JM, Ibrahim AE, Khalifian S, et al. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and molecular aspects, current challenges, and novel concepts. *Front Immunol* 2013;4:406.
26. Cendales LC, Kanitakis J, Schneeberger S, Burns C, Ruiz P, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. *Am. J Transplant* 2008;8:1396-1400.
27. Fleissig YY, Beare JE, LeBlanc AJ, Kaufman CL Evolution of the rat hind limb transplant as an experimental model of vascularized composite allotransplantation: Approaches and advantages. *SAGE Open Med* 2020;8:2050312120968721.
28. Meuli-Simmen C, Eiman T, Alpert BS, Meyer VE, Buncke GM, et al. Fibromuscular proliferation in finger arteries after hand replantation: a case report. *Microsurgery* 1996;17:551-554.
29. Griffin PA, Tan E, Katsaros J Late arterial thrombosis in a replanted thumb: a case report. *J Reconstr Microsurg* 1989;5:75-77.
30. Etra JW, Shores JT, Sander IB, Brandacher G, Lee WPA Trauma-induced Rejection in Vascularized Composite Allotransplantation. *Ann Surg* 2020;271:e113-e114.
31. Goutard M, Lellouch AG, Dussol B, Lantieri LA Facial Trauma 8 years after a Face Transplantation. *Plast Reconstr Surg Glob Open* 2021;9:e3575.

32. Kanitakis J, Petruzzo P, Gazarian A, Karayannopoulou G, Buron F, et al. Capillary Thrombosis in the Skin: A Pathologic Hallmark of Severe/Chronic Rejection of Human Vascularized Composite Tissue Allografts? Transplantation 2016;100:954-957.

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Mimicking Clinical Rejection Patterns in a Rat Osteomyocutaneous Flap Model of Vascularized Composite Allotransplantation

Short Title: Rat VCA model of vasculopathy

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Authorship

A.L., J.H., and C.K. conceived and designed the research project. J.B., Y.F., R.R., and N.K. performed the surgeries and performed daily operations of the experiments. J.B., Y.F., and N.K. prepared the tissue samples, performed histological staining, and imaged them. J.B. performed histological measurements and statistical analyses of the data. J.B., Y.F., R.R., N.K., A.L., J.H., and C.K. wrote the manuscript.

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Abbreviations

ACR, acute cellular rejection

CR, chronic rejection

EEL, external elastic lamina

IEL, internal elastic lamina

IRI, ischemia reperfusion injury

MHC, major histocompatibility complex

POD, post-operative day

SOT, solid organ transplant

VCA, vascularized composite allotransplantation

Abstract

Background: Graft loss in vascularized composite allotransplantation (VCA) is more often associated with vasculopathy and chronic rejection (CR) than acute cellular rejection (ACR). We present a rat osteomyocutaneous flap model using titrated tacrolimus administration that mimics the graft rejection patterns in our clinical hand transplant program. Comparison of outcomes in these models support a role for ischemia reperfusion injury (IRI) and microvessel changes in chronic rejection of skin and large vessel vasculopathy. The potential of the surgical models for investigating mechanisms of rejection and vasculopathy in VCA and treatment interventions is presented.

Materials and Methods Four rodent groups were evaluated: syngeneic controls (Group 1), allogeneic transient immunosuppression (Group 2), allogeneic suboptimal immunosuppression (Group 3), and allogeneic standard immunosuppression (Group 4). Animals were monitored for ACR, vasculopathy, and CR of the skin.

Results Transient immunosuppression resulted in severe ACR within two weeks of tacrolimus discontinuation. Standard immunosuppression resulted in minimal rejection but subclinical microvessel changes, including capillary thrombosis and luminal narrowing in arterioles in the donor skin. Further reduction in tacrolimus dose led to femoral vasculopathy and CR of the skin. Surprisingly, femoral vasculopathy was also observed in the syngeneic control group.

Conclusions Titration of tacrolimus in the allogeneic VCA model resulted in presentations of rejection and vasculopathy similar to those in patients and suggests

vasculopathy starts at the microvessel level. This adjustable experimental model will allow the study of variables and interventions, such as external trauma or complement blockade, that may initiate or mitigate vasculopathy and CR in VCA.

Key Words

Rat osteomyocutaneous flap model, transplantation, vascularized composite allotransplantation, rejection, vasculopathy

Introduction

Clinical experience with vascularized composite allotransplantation (VCA) of the upper extremity is now in its third decade; results to date have demonstrated good outcomes with complications similar to those in solid organ transplantation (SOT(1)). In contrast to the fairly homogenous tissue composition of SOT, upper extremity and some face VCA are composed of skin, muscle, bone, and other tissues, each with its own immunogenicity characteristics. More than 80% of VCA patients exhibit acute rejection events within the first year following transplant(2, 3). This high rate of rejection has been attributed to the immunogenicity of the skin(4-6), but may also be related to the ease of diagnosing rejection in an allograft that can be visualized directly(5). Lung transplants that are directly exposed to the environment also have high rates of rejection(7), albeit not as high as those observed in VCA recipients.

Historically, graft rejection was broadly defined as acute rejection, either of a cellular or humoral nature, or chronic rejection, an indolent process that can progress to obliterative vasculopathy and parenchymal fibrosis(8, 9). The risk of acute rejection is high in the first few months post-transplant, while chronic rejection occurs months to

years post-transplant. Exposure of the recipient to allogeneic MHC on donor antigen presentation cells (APC) or direct allorecognition may result in a stronger T cell activation and response early post-transplant(10), while recipient exposure to donor MHC presented on the host APC (indirect presentation) leads to activation of a more limited T cell repertoire and graft targets, resulting in chronic rather than acute rejection processes(11). The definition of the different types of rejection is an ongoing and revolving process and is graft specific. Recently, the European Society of Organ Transplantation published an update on the current six different histological phenotypes of acute and chronic rejection in kidney transplantation(12). The definition of chronic rejection in VCA recipients is also being defined(13), and donor vasculopathy resulting in ischemic graft loss and fibrotic changes in the skin have been described(4, 14, 15). Vasculopathy in VCA is similar to that observed in cardiac transplantation, defined as diffuse stenosis caused by concentric intimal hyperplasia and inadequate compensatory outward modeling(16). Like VCA grafts(14, 17), all vessels may be involved, from the microcirculation to the large epicardial arteries and the coronary veins(18). Presentation occurs months to years after transplantation, indicating a chronic vs. acute immune response. While lesions are fairly uniform, the progression to vessel narrowing and eventually allograft ischemia(19-21) is quite focal(18). In addition to chronic rejection in the form of vasculopathy, VCA allografts have multiple CR targets, including skin and adnexal structures (hair follicles, sebaceous glands, nailbeds) as well as vessels(13).

While acute vs. chronic rejection can be somewhat defined by the temporal relation to time of transplant, acute cellular or humoral rejection can occur any time post-transplant. Acute rejection is differentiated from chronic rejection by a more

intense mononuclear cell infiltrate in parenchymal tissue and can result in significant and even necrotic tissue damage. Acute humoral rejection is also referred to as acute vascular rejection; this form of rejection is not associated with cellular infiltrates and is characterized by focal ischemia, endothelial swelling and intravascular coagulation(22). Chronic rejection of the skin is associated with minimal cellular infiltrate, with extensive fibrosis and loss of adnexal structures (i.e. hair follicles and sebaceous glands) and normal skin architecture. Multiple presentations of chronic rejection have been documented in VCA patients, including ischemic vasculopathy in the absence of ACR or skin involvement, CR of the skin, and loss of fingernails with minimal vasculopathy as well as severe ACR, vasculopathy, and chronic skin changes(13).

There remains much speculation regarding the pathogenesis of vasculopathy; factors such as inadequate immunosuppression, extensive physical activity of the hand, vibrational injury or other traumatic mechanical damage, infection, ischemia-reperfusion injury, and cell- and antibody-mediated rejection have been proposed(4, 13-15, 17, 20). The common mechanisms and presentation of acute and severe vasculopathy and CR in the skin vs. non-progressive vasculopathy between VCA recipients have yet to be elucidated.

These unanswered questions regarding the targets and presentation of CR in hand transplant and other VCA patients necessitate the development of animal VCA models that will allow for the intervention or exacerbation of ACR, vasculopathy, and skin CR during a clinically relevant course of immunosuppression. Such models will offer insights into rejection and vasculopathy in the face of interventions such as complement blockade, infectious challenge, and vibrational or other traumatic injuries.

In this study, we utilized a modified rat heterotopic osteomyocutaneous flap hind limb allograft(23) to develop an adaptable model of VCA with carefully titrated immunosuppression which allows for the study of ACR and CR of the skin, as well as vasculopathy, as seen in a clinical setting.

Materials and Methods

Animals

Complete major histocompatibility complex (MHC)-mismatched male Brown Norway (BN, RT1.Aⁿ) and Lewis (Lew, RT1.A^l) rats aged 12-16 weeks and weighing 275-350 grams were used as VCA donors and recipients, respectively. A cohort of Lew rats was used as syngeneic donors. Animals were obtained from Charles River Laboratories (Wilmington, MA, USA) and acclimated to the animal facility for at least one week prior to surgery. The procedures were performed in accordance with the University of Louisville Institutional Animal Care and Use Committee (IACUC protocol #18198) and NIH *Guide for the Care and Use of Laboratory Animals*(24).

Donor Surgery

Donor osteomyocutaneous flap grafts were recovered for implantation as previously described(23). Briefly, the femoral vessels of donor BN (allograft) or Lew (syngeneic) animals were mobilized by ligating and cutting all vessels other than those providing arterial flow and venous return from the graft, which consisted of the tibia, fibula, surrounding muscles, nerves, and skin. The knee and ankle joints were disarticulated and the artery and vein pedicles were cut proximally near the inguinal

ligament. One milliliter of heparinized saline (30 Units/ml) was injected into the artery to flush the graft. This volume was sufficient to flush all blood from the graft with clear saline effluent post-flush. Recent experience of graft failure with increased cold ischemia times have resulted in our increasing the amount of flush used to 4 ml. For the experiments presented here, 1 ml of heparinized saline was used. After graft recovery, the donor rat was euthanized by pneumothorax.

Recipient Surgery

Recipient animals were anesthetized using 2.5% isoflurane via an endotracheal tube and then prepared for graft transplantation as previously described(23). Donor vessels were anastomosed venous end-to-side, arterial end-to-end using 10-0 nylon interrupted sutures. Visual assessment of the anastomoses confirmed effective graft reperfusion. The donor and recipient surgeries were performed concurrently and the total ischemic time for the donor limb flap was 60-90 minutes during which the graft was at room temperature while anastomoses were performed. The graft was inserted into the inguinal pocket and oriented upside down, with the ankle joint superior and the knee joint inferior. Upon removal from anesthesia, the rats were placed on a heating pad for thermal support, administered meloxicam (4 mg/kg body weight) subcutaneously for pain suppression, and monitored closely until recovery. Additional meloxicam doses were administered daily until postoperative day (POD) 3.

Immunosuppressive Regimens

A schematic diagram of the four groups and dosing regimens is shown in Fig 1. Group 1 syngeneic Lewis recipients (n=3) did not receive immunosuppression throughout the experiment, emulating clinical replant recipients without alloreactivity, and were monitored daily and explanted on POD60-POD66. Allogeneic recipients in Groups 2-4 were weighed twice weekly and administered daily subcutaneous injections of 2 mg/kg tacrolimus until POD11. In the Group 2 transient immunosuppression model, which was designed to produce early aggressive ACR, immunosuppression was discontinued after POD11; animals were monitored daily until POD41-POD44, when rejection necessitated explants (n=5). The Group 3 suboptimal Immunosuppression group was designed to produce a common presentation of chronic skin changes with large vessel vasculopathy, achieved via a stepwise decrease in immunosuppression after POD11, first to 0.5 mg/kg tacrolimus (POD12-POD26) and then to 0.25 mg/kg tacrolimus from POD27 until termination at around three months or in the case of graft failure. Termination in this group occurred between POD68 and POD97 (n=5). Finally, the Group 4 standard immunosuppression group was modeled on a clinical dose of tacrolimus known to maintain grafts with minimal ACR; tacrolimus was reduced after POD11 to 0.5 mg/kg until termination on POD60-POD64 (n=4).

VCA Recipient Monitoring

Recipient rats were housed singly and monitored daily for clinical signs of pain, dehydration, weight loss, and decreased activity, in addition to surgical failure (first 48-72h) or rejection. The initial clinical signs of rejection, including edema, maculopapular rash, erythema, and skin desquamation(25, 26) were recorded. Macroscopic images of

the grafted area were captured daily and scored for rejection by a double-blinded observer.

Laser Doppler Imaging

A Moor LD12-IR laser Doppler scanner (Moor Instruments, Wilmington, DE, USA) with an infrared 785 nm laser diode was used to assess blood perfusion through the graft skin on POD14 and the day of explant. Perfusion through the skin was measured on a colorimetric scale from no flow (blue) to high flow (red) (Fig. 2).

Tissue Biopsy and Explant Surgery

On POD14 and biweekly thereafter, recipient rats were anesthetized using 2.5% inhaled isoflurane, 2 mm skin biopsies were obtained, and the wound was closed with absorbable 4-0 suture. For explant, animals were deeply anesthetized with 5% inhaled isoflurane; larger biopsies of the donor-recipient skin interface were procured, as well as donor muscle biopsy. Arterial samples (1-2 mm in length) were obtained proximally (recipient side) and distally (donor side) to the anastomosis (Fig. 4A). All tissue samples were fixed in 10% formalin before paraffin embedding.

Histologic Evaluation

For morphometry, 6 μ m sections of skin, muscle, and vessel samples were stained with hematoxylin and eosin (H&E) using standard staining methods. Skin biopsies were examined for changes in skin morphology and architecture as well as

loss of adnexal structures (i.e., hair follicles and sebaceous glands). Skin and muscle biopsies were graded by a blinded observer, using the criteria listed in Table 1.

Evaluation of Graft Vasculopathy

All measurements were performed by an observer blinded to the sample location and immunosuppression group. The vessel lumen area as well as the internal and external elastic laminae (EEL) were traced using ImageJ analysis software (NIH, Bethesda, MD), allowing for the calculation of intimal and medial thickness and area. The total number of cell nuclei within the medial layer of each vessel was determined. Percent intimal proliferation was calculated as intimal thickness/total laminae thickness, and the occlusion ratio was calculated as lumen diameter/total vessel diameter. For skin microvessel analysis, the total number of microvessels (defined as vessels with a lumen diameter of less than 200 μm) was determined for the donor skin area as well as the recipient skin area. The percentage of microvessels occluded by thrombosis, lumen narrowing, and/or microvessel wall thickening was calculated separately for donor and recipient skin areas.

Statistical Analyses

Statistical analyses were performed using SigmaPlot 14.0 (Systat, San Jose, California, USA) with $p < .05$ significance level. Within group and within location (donor, recipient, and contralateral artery) one-way ANOVA was performed on all histological vessel measurements, followed by Holm-Sidak *post hoc* analyses where appropriate.

To ensure rigor, histological images were coded and analyzed in a blind fashion with the code being broken only after all scoring quantifications were acquired.

Results

Operative results

The donor operative time was approximately 45 min, while the recipient operative time was approximately 1.5 to 2 h. On rare occasions, minor postoperative complications, such as focal skin necrosis, usually on the caudal edge of the skin paddle, and iatrogenic skin necrosis following subcutaneous tacrolimus injections, were observed. Upon explant analysis, one animal in the standard immunosuppression group was found to have numerous masses in the inguinal cavity, peritoneal cavity, and lungs; this animal was excluded from histological analyses.

Graft Perfusion

Laser Doppler imaging of syngeneic animals revealed proper blood flow through the skin of the graft throughout the study. Macroscopic examination of the femoral artery revealed no signs of trauma or hypertrophy on either side of the anastomotic site (data not shown).

Group 2 transient immunosuppression animals exhibited sufficient perfusion to the graft on POD14, including robust recipient collateral feeding vessels (Fig. 2A). Blood perfusion to the skin was completely ablated on the day of explant (POD43)(Fig. 2B). Surgical exposure of the anastomoses during the explant revealed a healthy recipient

artery, whereas the donor artery distal to the anastomosis was severely inflamed and hypertrophied (Fig. 2C).

On POD14, Group 3 suboptimal immunosuppression animals presented with proper blood perfusion throughout the graft and abundant recipient collateral vessels (Fig. 2D), and terminal assessment revealed some areas of diminished blood flow within the VCA (Fig. 2E). Despite some loss of flow, macroscopic examination of the anastomoses did not indicate any obvious signs of vascular injury distal to the anastomosis (Fig. 2F).

In Group 4 standard immunosuppression animals, blood perfusion to the skin remained unchanged from POD14 (Fig. 2G) to POD64 (Fig. 2H), indicating sufficient blood flow throughout the course of the study. Exposure of the anastomoses at the explant revealed no outward signs of vascular trauma or thrombosis (Fig. 2I).

Clinical Signs of Rejection and Skin Histopathology

The Group 1 syngeneic control recipients exhibited normal healing, healthy skin components, and proper donor hair growth throughout the study. No clinical signs of rejection were noted. Biopsies from POD14 as well as at the time of explant (POD60-POD66) showed normal skin adnexal components. Interestingly, all the skin biopsies from Group 1 animals at two weeks showed grade1-2 cellular infiltrate (average histology score of 1.33, Table 1), with resolution of infiltrate by the time of explant (average skin histology score of 0.33, Table 1), possibly in response to IRI and surgical trauma.

In Group 2 transient immunosuppression animals, the skin showed normal wound healing and donor hair growth during the first 3 weeks post-surgery, indicating proper vascularization of the flap. The identification of donor skin vs. recipient skin was readily apparent because of the color and growth direction of the hair. The first signs of acute rejection, including skin redness, swelling, and partial loss of hair, were observed on $\text{POD}19.4 \pm 2.87$ days (Table 2); by the time of explant, severe acute rejection of donor skin and graft failure was evident in all animals (Fig. 3A). Skin histopathology from POD14 revealed normal morphology with healthy adnexa, hair follicles, and sebaceous glands; mild mononuclear cell infiltration was apparent (Grade 1-2 ACR score). In the explant, the donor skin lacked adnexal structures, and the epidermis was necrotic and atrophic (Fig. 3B). Microvessel thrombosis (Fig. 3C, white arrow) and extensive mononuclear cell infiltration were observed in the donor skin.

In Group 3 animals receiving suboptimal immunosuppression, clinical follow-up showed a normal VCA skin appearance with healthy hair growth on POD14. The identification of donor vs. recipient skin was readily apparent. Skin biopsies on POD14 showed normal morphology with diffuse mononuclear cell infiltration (Grade 2 ACR score, Table 1). When immunosuppression therapy was decreased to 0.25 mg/kg, rejection events including maculopapular lesions, gradual hair loss, and atrophy of donor muscle and skin were noted; the first rejection event was observed at $\text{POD}39.8 \pm 3.57$ days (Table 2). The donor skin over the flap gradually reduced in size over the course of the experiment and was eventually covered with a new epidermis (Fig. 3D). In explants, mononuclear cell infiltration was apparent in the donor skin (Fig. 3E), but to a lesser extent than that observed in ACR animals. Histopathology revealed a lack of

adnexal structures in the donor skin, with severely contracted fibrotic donor skin with loss of the rete ridges and epidermal thinning. New skin likely of recipient origin (hair growth in this area was white in color suggesting Lewis strain origin) covered the rest of the muscle and bone graft. The recipient skin exhibited healthy adnexal structure, epidermis, and vessel components. At greater magnification, arteriolar wall thickening with perivascular infiltration (Fig. 3F, black arrow) was observed in the donor area of the skin.

In the Group 4 standard immunosuppression group, clinical follow-up revealed normal VCA skin and hair throughout the study (Fig. 3G). When immunosuppression was maintained at 0.5 mg/kg, the recipient animals did not exhibit gross signs of rejection. One animal in this group showed redness and swelling on POD44. However, this animal developed tumors throughout the graft and in the peritoneal cavity and lungs, necessitating its removal from the study. Skin biopsies on POD14 showed no signs of adnexal structure loss with minimal mononuclear cell infiltration (primarily ACR Grade 1), similar to both rejection groups (Table 1). Skin biopsy of the recipient/donor graft border at the explant revealed that the donor skin resembled the recipient skin. The skin exhibited normal epidermis and adnexa without morphological change, and mild mononuclear infiltration (Fig. 3H). At a higher magnification, occasional microvascular thrombosis (white arrow) and vessel wall thickening (black arrow) were observed in the donor skin (Fig. 3I).

Vessel Histopathology and Quantitative Measurement

Samples of the femoral artery proximal (recipient) and distal (donor) to the anastomosis were obtained (Fig. 4A). Histological analyses of the Group 1 syngeneic donor, recipient, and contralateral artery samples revealed no differences in either the total number of cells (Fig. 6A) or cells/ μm^2 (Fig. 6B) in the intimal and medial laminae of the vessel. The donor lumen area was slightly, but not significantly, constricted (Fig. 6C). A layer of apoptotic cells lined the lumen along the intimal interface on both the recipient and donor sides of the anastomosis. No differences were observed in intimal thickness (Fig. 6D), medial thickness (Fig. 6E), total lamina thickness (Fig. 6F), intimal proliferation (Fig. 7A), occlusion ratio (Fig. 7B), percentage of occluded skin microvessels (Fig. 7C), or skin microvessels/ mm^2 (Fig. 7D).

In the Group 2 transient immunosuppression animals, designed to develop severe acute rejection of the skin, the femoral artery exhibited changes consistent with necrotic rejection rather than vasculopathy. A robust mononuclear cell invasion in the medial layer of the donor artery (Fig. 4E) was detected compared to that in the recipient artery (Fig. 4B). An apoptotic layer of cells lining the lumen of the distal artery sample was observed (Fig. 4E), with pronounced microvascular thrombosis in the adventitial layer (Fig. 4E; examples shown with black arrows). Severe vasculitis of the donor artery in this group required imaging of the vessel sample at a lower magnification (10x); all other vessel images in Fig. 4 were acquired at 20x magnification. Quantitative vessel measurements did not show significant differences in total cells (Fig. 6A) and cells/ μm^2 (Fig. 6B) in the intimal+medial laminae. The lumen area (Fig. 6C) and intimal thickness (Fig. 6D) were unchanged, with increased medial thickness in the donor artery distal to the anastomosis compared to contralateral vessel samples (one-way ANOVA, DF=2,

F=8.081, $p=.006$, Holm-Sidak *post hoc* $p=.005$)(Fig. 5E). Similarly, the total laminae thickness (intimal+medial layers) exhibited significant outward remodeling in the donor artery compared to the contralateral control vessel (one-way ANOVA, $DF=2$, $F=6.090$, $p=.015$, Holm-Sidak *post hoc* $p=.016$. Intimal proliferation and occlusion ratios were unchanged between donor, recipient, and contralateral vessel samples (Fig. 7A and 7B, respectively). Significantly more donor skin capillaries were occluded compared to syngeneic donor skin (one-way ANOVA, $DF=3$, $F=9.785$, $p=.001$, Holm-Sidak *post hoc* $p=.001$)(Fig. 7C).

Group 3 was designed to replicate chronic rejection patterns seen in clinical VCA recipients, including chronic skin rejection with fibrosis and loss of adnexal structures and rete ridges as well as intimal hyperplasia and medial hypertrophy or graft vasculopathy in the donor vessels. In Group 3 suboptimal immunosuppressed animals, vessel samples from the recipient side of the femoral artery anastomosis exhibited healthy intimal and medial layers (Fig. 4C). Marked intimal hyperplasia was apparent in the donor artery (Fig. 4F, yellow bracket) and apoptotic cells lined the innermost layer of the intima along the lumen. Significantly more infiltrating cells/ μm^2 of the intimal+medial area was found (one-way ANOVA, $DF=2$, $F=6.092$, $p=.015$) both in the recipient ($p=.023$) and donor arteries ($p=.031$) than in the contralateral arteries (Fig. 6B). The lumen area was unchanged (Fig. 6C), while the intima was significantly thicker in the donor vessel than in the contralateral control artery (one-way ANOVA, $DF=2$, $F=5.747$, $p=.018$, Holm-Sidak *post hoc* $p=.016$)(Fig. 6D). The medial thickness and total lamina thickness remained unchanged (Fig. 6E and 6F, respectively). Intimal proliferation was significantly higher in donor vessels (one-way ANOVA, $DF=2$, $F=13.310$, $p<.001$) than in

both recipient ($p=.005$) and contralateral arteries ($p=.001$)(Fig. 7A). The occlusion ratio was unchanged (Fig. 7B), while the percentage of occluded skin microvessels was significantly higher in the donor skin than in Group 1 syngeneic donor skin (one-way ANOVA, $DF=3$, $F=9.785$, $p=.001$, Holm-Sidak *post hoc* $p=.004$)(Fig. 7C). Skin microvessels/mm² remained unchanged (Fig. 7D).

Groups 2-4 all exhibited normal vessel components on the recipient side of the anastomosis (Fig. 4B,4C and 4D). Unexpectedly, 2/3 animals in Group 1 showed mild intimal hyperplasia on the recipient side of the anastomosis (Fig. 5C, 5D). In addition, the syngeneic control animals showed moderate to severe intimal hyperplasia in 2/3 animals on the donor side of the anastomosis (Fig. 5E, 5F). Evidence of intimal hyperplasia was observed in the donor vessel of Group 4 (Fig. 4G), with a layer of apoptotic cells lining the innermost layer of the intima, but not to the extent seen in Group 3 animals who showed overt intimal hyperplasia in the donor vessel (Fig 4F). Group 2 animals who received transient immunosuppression that resulted in severe necrotic acute rejection had donor arteries with extensive lymphocytic infiltration and capillary thrombosis of the adventitial layer, and acentric intimal and medial proliferation consistent with severely inflamed tissue (Fig. 4E).

In addition to skin histology, we also scored biopsies of muscle taken at time of explant. Muscle showed cellular infiltrate and evidence of tissue damage in a similar pattern as seen for skin biopsies taken at explant. Biopsies were scored as defined in Table 1, and showed mild to diffuse cellular infiltrate in Group 1 syngeneic animals, and Group 4 standard Immunosuppression. As expected, muscle at explant from animals with severe ACR (Group 2) revealed robust cellular infiltration, lack of cross striation

and hypochromagenicity of the muscle, and arteriole hyperplasia (black arrow, Fig. 8A) (Table1). Muscle biopsy from Group 3 Suboptimal Immunosuppression animals revealed moderate cellular infiltration and decreased lumen diameter in the muscle arterioles (Fig. 8B, black arrows) (Table 1). In contrast, Histological analysis of the explant muscle samples from Group 4 standard immunosuppression animals revealed mild cell infiltration and healthy microvessels (Fig. 8C, white arrow) (Table 1).

Discussion

Multiple rodent experimental models of VCA have been described, each with its own advantages and drawbacks(27). The model presented here was developed at our center(23) and offers key components that mimic various presentations of rejection observed in the clinic. The groups presented were successful in modeling patterns seen in clinical replant patients (Group 1 – Syngeneic transplants in the absence of immunosuppression), and in clinical VCA transplant recipients including ACR without vasculopathy (Group 2 – Transient immunosuppression), evidence of both chronic skin changes and vasculopathy (Group 3 – Suboptimal immunosuppression), and minimal ACR with some microvessel thickening in the absence of significant large vessel involvement or significant acute chronic skin rejection (Group 4 – Standard immunosuppression).

The rejection spectrum demonstrated by the groups in this study is influenced by four variables: IRI/surgical trauma, allogeneic donor MHC, the level of immunosuppression and duration of immunosuppression. Group 1 animals showed an unexpected amount of intimal proliferation on both the recipient and donor side of the

anastomosis. Of relevance to the observation in this report of vasculopathy in the donor vessel of our syngeneic control groups, vasculopathy can also occur when digits and upper extremities are replanted following amputation(28, 29). This vasculopathy is not related to alloantigen and likely originates in the ischemia reperfusion injury (IRI) to the replanted tissue as a result of the trauma and surgical procedure.

A characteristic shared by Group 1 and clinical replant patients is that neither received tacrolimus immunosuppression. As we did not see significant hyperplasia on the recipient side of the anastomosis in Group 2-4, nor in the donor femoral artery in Group 2 animals, it may be that the tacrolimus interfered with the development of non-allogeneic related intimal hyperplasia. A future direction of ours is to repeat Group 1 and also test the ability of tacrolimus to interfere with the development of intimal hyperplasia due to IRI and surgical trauma. In the Group 2 transient immunosuppression group, when immunosuppression was halted 12 days postoperatively, severe acute rejection events occurred in less than two weeks, resulting in necrotic compromise of the skin and ischemic changes before vasculopathy could develop. This model mimics the rare non-compliance situation where patients stop taking immunosuppression. In the Group 3 suboptimal immunosuppression group, tacrolimus administration was extended but reduced in a stepwise fashion and maintained at lower doses until terminal time points, which allowed both chronic skin rejection with loss of adnexal structures and moderate to severe vasculopathy to develop. In the Group 4 standard immunosuppression animals, the tacrolimus dose was lowered on POD12 but maintained at a dose sufficient to keep the graft outwardly healthy. On the surface, the skin and hair appeared normal throughout the course of the study and blood perfusion was uniform throughout the flap

at the explant. This group models patients on standard immunosuppression that are doing well clinically, but over time develop histologic changes in skin biopsies and mild intimal hyperplasia of donor vessels. In Group 4 animals, skin and vessel histology at explant showed scattered microvessel narrowing and thrombosis and mild medial hyperplasia of the donor femoral artery distal to the anastomosis. We expect mild intimal hyperplasia would also develop if animals were maintained longer. This presentation is most similar to the clinic, where the target is the lowest amount of immunosuppression that will maintain the graft, while minimizing immunosuppression complications. While Group 4 models most transplant recipients with good graft function but evidence of ongoing low-level rejection in the skin and vessels, it does not model the confluent aggressive ischemic vasculopathy and graft loss in the absence of skin rejection seen in our fourth transplant recipient(20). We hypothesize that this type of rejection is a combination of alloreactivity and external physical trauma to the graft. We have evidence that vibrational trauma can exacerbate vasculopathy in this rat model of VCA (unpublished data), and other groups have demonstrated the potential role of physical and thermal trauma in promoting graft rejection in VCA recipients(30, 31).

These models will be used in experiments to test potential interventions or define mechanisms that result in graft rejection. VCA patients that are doing well clinically but have histologic evidence of alloimmune-mediated damage are vulnerable to progression from a number of causes. Reduced doses of immunosuppression occur clinically because of related complications, neoplasia, or compliance issues. In addition, we expect accumulation of cellular- and antibody-mediated rejection events, infection,

and/or physical trauma will promote progression to chronic rejection of the skin and vasculopathy in our animals.

In the Group 4 standard immunosuppression model, it appears the vascular injury in the graft begins in the small vessels. Microvessels in the donor skin exhibited thrombosis and lumen narrowing, with minimal signs of vasculopathy in the donor femoral artery. This observation may indicate a cascade of vascular involvement in rejection that begins in the smallest vessels. Strengthening this argument, Kanitakis et al. have observed capillary thrombosis prior to vasculopathy that ultimately led to the loss of two grafted fingers in a VCA patient(32). While multiple mechanisms have been proposed for developing vasculopathy, few studies have focused on the initiating events, and provide evidence that the microvessels are affected first, as seen in Group 4.

It is important to consider the limitations and opportunities for development of the current study. While the analysis of acute vs. chronic rejection of the skin was based on mononuclear cell infiltrate and patterns of damage and fibrosis by H&E, as is done clinically, we performed staining of CD3, CD45RA and several other lineage markers which produced expected staining patterns. However, the background staining was unexpectedly high, despite multiple attempts to optimize with different blocking agents. We suspect that fixation in 10% formalin for extended periods may have contributed to the high backgrounds and this data could not be included. Additionally, two other clinical presentations of rejection seen on our hand transplant recipients remain to be developed. First is a model of development of chronic skin changes in the absence of significant vasculopathy, which has also been observed in VCA patients(13).

Additionally, we are working on a model that mimics aggressive confluent ischemic vasculopathy without ACR in the skin that resulted in early graft loss in our fourth hand transplant recipient(20).

Conclusions

Here, we present a reliable and reproducible tool for studying the underlying mechanisms of VCA graft vasculopathy and acute and chronic rejection of the skin. Careful titration of immunosuppression in this model can push a graft towards different levels of rejection, allowing for later administration of additional graft stressors (i.e. infection, mechanical insult, etc.) or interventions such as complement blockade to ascertain potential thresholds that could ultimately identify mechanisms and potential for prevention or treatment of skin CR and vasculopathy.

Figure Legends

Figure 1. Schematic diagram of the tacrolimus dosing regimens utilized to in control (Group 1) and experimental groups (Groups 2-4).

Figure 2. Laser Doppler imaging of a representative animal from each experimental group, with the border of the skin window marked by white dashed lines. Using transient immunosuppression (Group 2), robust perfusion through the graft as well as recipient collateral superficial blood supply was observed at POD14 (A) followed by complete loss of blood flow at POD43 (B). Exposure of the anastomosis (C, white arrow) at time of explant revealed severe vascular inflammation and edema distal to the anastomosis (C, black arrow). With suboptimal immunosuppression (Group 3), blood flow to the graft was normal at POD14 (D); by explant at POD90, nearly half of the graft exhibited loss of blood flow (E). The femoral artery appeared normal on both sides of the anastomosis (F, white arrow). With standard immunosuppression (Group 4), blood perfusion was normal throughout the entirety of the graft both at POD14 (G) and at explant on POD64 (H). The femoral artery exhibited normal morphology near the anastomosis site (I, white arrow).

Figure 3. Skin histopathology. At time of explant, Group 2 transient immunosuppression animals exhibited necrotic skin over the graft (A). Skin samples taken at the donor-recipient skin border (A, black box) showed massive mononuclear cell infiltration on both sides of the border, and necrotic epidermis as well as loss of skin adnexal

components on the donor side (B); further magnification of donor skin (B, black box) showed perivascular infiltration as well as microvascular thrombosis (C, white arrow). Group 3 suboptimal immunosuppression animals exhibited pervasive hair loss and contraction of donor skin consistent with chronic rejection of the skin (D). A skin sample traversing donor-recipient-donor skin border revealed moderate mononuclear cell infiltrate localized to the donor tissue (E). A healthy new layer of epidermis was observed over the donor skin component of the graft; further magnification of donor skin (E, black box) revealed perivascular infiltration and narrowing of the arteriole lumen (F, black arrow). Animals in the standard immunosuppression group exhibited normal skin and hair growth throughout the entirety of the study (G). A skin sample taken at the donor-recipient skin border (G, black box) revealed normal skin on both sides of the graft, including healthy epidermis and normal skin adnexal components (H). Further magnification (H, black box) indicated vessel wall thickening of a microvessel (I, black arrow) and microvascular thrombosis (I, white arrow). Scale bar for B, E, and H = 500 μm ; scale bar for C, F, and I = 100 μm .

Figure 4. Femoral artery histopathology. Samples of the recipient femoral artery and donor femoral artery were obtained proximal and distal to the anastomosis site, respectively (A). In groups 2-4, recipient samples exhibited a thin intimal layer (yellow arrows) and comparable medial (red brackets) and adventitial layers (blue brackets)(B-D). Acute Rejection animals showed massive mononuclear cell infiltration in both the media (red bracket) and adventitia (blue bracket), as well as microvascular thrombosis of the *vaso vasorum* (black arrows show several examples), but no significant intimal

hyperplasia (E). Suboptimal immunosuppression animals exhibited marked intimal hyperplasia (yellow bracket) with a layer of apoptotic endothelial cells lining the lumen, but relatively unchanged media (red bracket) and adventitia (blue bracket)(F). Group 4 standard immunosuppression animals exhibited signs of apoptosis of the endothelial lining and intima (yellow bracket), with slight outward remodeling of the media (red bracket) and unchanged adventitia (blue bracket)(G). Scale bar = 100 μ m for all panels; images were captured at 20x magnification for all panels except E, which was captured at 10x magnification.

Figure 5. Femoral artery histopathology of Group 1 syngeneic control animals. Samples of the recipient femoral artery and donor femoral artery were obtained proximal and distal to the anastomosis site, respectively (A). Unlike Groups 2-4, two of the three animals in Group 1 showed some level of intimal hyperplasia on the recipient side of the anastomosis. (C,D). In addition, microvascular thrombosis of the *vaso vasorum* (black arrows show several examples) were seen in the adventitia of the recipient side sample in the third animal (D). When the donor side of the femoral artery was examined, again, two of the three animals showed significant intimal proliferation, even more than seen on the recipient side of the anastomosis (E,F). The third animal showed no intimal proliferation and normal histology of the donor femoral artery (G). Scale bar = 100 μ m for all panels; images were captured at 20x magnification for all panels.

Figure 6. Quantification of total nuclei in the intimal+medial laminae (A), nuclei/ μm^2 in intimal+medial laminae (B), lumen area (C), intimal thickness (D), medial thickness (E), and total laminae thickness (F) from femoral artery histological samples. All graphs display group means + standard error. * $p < .05$ when compared to contralateral artery sample from the same group. Transient immunosuppression was characterized by cell infiltration to the donor vessel, increased medial thickening and increased total laminae area due to outward remodeling, whereas suboptimal immunosuppression resulted in cell infiltration to both donor and recipient vessel as well as intimal hyperplasia. Standard immunosuppression resulted in no large vessel differences compared to the animals' unoperated control vessel or syngeneic surgical controls.

Figure 7. Quantification of intimal proliferation (A), occlusion ratio (B), and percentage of skin microvessels occluded (C). All graphs display group means + standard error. * $p < .05$ when compared to contralateral vessels from the same group, # $p < .05$ when compared to syngeneic samples, ^ $p < .05$ when compared to recipient vessel samples from the same group. All groups exhibited significantly higher percentage of occluded microvessels in donor skin compared to syngeneic controls.

Figure 8. Representative images of muscle biopsy at the time of explant collection for each group. Animals in the transient immunosuppression (Group 2) exhibited robust mononuclear cell infiltration as well as hyperplasia of capillary vascular structures (A, black arrow). Group 3 suboptimal immunosuppressed animals showed moderate cell

infiltration, combined with compromised lumen diameter in muscle capillaries (B, black arrows). Group 4 standard immunosuppression resulted in minimal cell infiltration, healthy muscles, and normal capillaries (C, white arrow). Scale bar = 100 μ m.

Table 1. Rejection scoring for skin and muscle biopsies. All samples taken from Allogeneic transplants (Groups 2-4) at POD14 exhibited mild cell infiltration. Surprisingly Group 1 syngeneic recipients showed mild infiltrate at two weeks, which resolved by the time of explant. At explant, severe ACR with major epidermal necrosis was seen in Group 2, moderate infiltrate with some epidermal loss in Group 3 suboptimal immunosuppression animals, and mild to moderate infiltrate in Group 4 standard immunosuppression animals. Muscle biopsies at explant showed similar patterns, with mild cell infiltrate in Group1 syngeneic animals, heavy mononuclear cell infiltration with myocyte necrosis in Group 2 transient Immunosuppression animals, moderate cell infiltrate in Group 3 animals, and mild/diffuse cell infiltrate with minimal tissue damage in Group 4 standard immunosuppression animals.

Table 2. Gross clinical observation of graft. A t-test on time to first rejection event revealed significantly delayed rejection events in Group 3 suboptimal Immunosuppression animals compared to Group 2 transient immunosuppression animals (*p=.002).

References

1. Kaufman CL, Bhutiani N, Ramirez A, Tien HY, Palazzo MD, et al. Current Status of Vascularized Composite Allotransplantation. *Am Surg* 2019;85:631-637.
2. Petruzzo P, Lanzetta M, Dubernard JM, Landin L, Cavadas P, et al. The International Registry on Hand and Composite Tissue Transplantation. *Transplantation* 2010;90:1590-1594.
3. Kueckelhaus M, Fischer S, Seyda M, Bueno EM, Aycart MA, et al. Vascularized Composite Allotransplantation: Current standards and novel approaches to prevent acute rejection and chronic allograft deterioration. *Transpl Int* 2016;29:655-662.
4. Kollar B, Kamat P, Klein HJ, Waldner M, Schweizer R, et al. The Significance of Vascular Alterations in Acute and Chronic Rejection for Vascularized Composite Allotransplantation. *J Vasc Res* 2019;56:163-180.
5. Kaufman CL, Marvin MR, Chilton PM, Hoying JB, Williams SK, et al. Immunobiology in VCA. *Transpl. Int* 2016;29:644-654.
6. Kadono K, Gruszynski M, Azari K, Kupiec-Weglinski JW Vascularized composite allotransplantation versus solid organ transplantation: innate-adaptive immune interphase. *Curr Opin Organ Transplant* 2019;24:714-720.
7. Costa J, Benvenuto LJ, Sonett JR Long-term outcomes and management of lung transplant recipients. *Best Pract Res Clin Anaesthesiol* 2017;31:285-297.
8. Libby P, Pober JS Chronic rejection. *Immunity* 2001;14:387-397.
9. Demetris AJ, Murase N, Lee RG, Randhawa P, Zeevi A, et al. Chronic rejection. A general overview of histopathology and pathophysiology with emphasis on liver, heart and intestinal allografts. *Ann Transplant* 1997;2:27-44.
10. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, et al. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* 1997;64:795-800.
11. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, et al. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* 2001;167:7199-7206.
12. Becker JU, Seron D, Rabant M, Roufosse C, Naesens M Evolution of the Definition of Rejection in Kidney Transplantation and Its Use as an Endpoint in Clinical Trials. *Transpl Int* 2022;35:10141.
13. Kaufman CL, Kanitakis J, Weissenbacher A, Brandacher G, Mehra MR, et al. Defining chronic rejection in vascularized composite allotransplantation-The American Society of Reconstructive Transplantation and International Society of Vascularized Composite Allotransplantation chronic rejection working group: 2018 American Society of Reconstructive Transplantation meeting report and white paper Research goals in defining chronic rejection in vascularized composite allotransplantation. *SAGE Open Med* 2020;8:2050312120940421-2050312120940421.

14. Morelon E, Petruzzo P, Kanitakis J Chronic rejection in vascularized composite allotransplantation. *Curr Opin Organ Transplant* 2018;23:582-591.
15. Krezdorn N, Lian CG, Wells M, Wo L, Tasigiorgos S, et al. Chronic rejection of human face allografts. *Am J Transplant* 2019;19:1168-1177.
16. Mitchell RN, Libby P Vascular remodeling in transplant vasculopathy. *Circ. Res* 2007;100:967-978.
17. Kanitakis J, Petruzzo P, Badet L, Gazarian A, Thaunat O, et al. Chronic Rejection in Human Vascularized Composite Allotransplantation (Hand and Face Recipients): An Update. *Transplantation* 2016;100:2053-2061.
18. Laks JA, Dipchand AI Cardiac allograft vasculopathy: A review. *Pediatr Transplant* 2022;26:e14218.
19. Diefenbeck M, Nerlich A, Schneeberger S, Wagner F, Hofmann GO Allograft vasculopathy after allogeneic vascularized knee transplantation. *Transpl. Int* 2011;24:e1-e5.
20. Kaufman CL, R O, BW B, JE K, Tsai TM, et al. Graft Vasculopathy in Clinical Hand Transplantation. *Am. J Transplant* 2012;12:1004-1016.
21. Shetty M, Chowdhury YS Heart Transplantation Allograft Vasculopathy. *StatPearls. Treasure Island (FL):* 2021.
22. Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, et al. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999;10:2208-2214.
23. Fleissig Y, Reed RM, Beare JE, LeBlanc AJ, Williams SK, et al. Modified Heterotopic Hindlimb Osteomyocutaneous Flap Model in the Rat for Translational Vascularized Composite Allotransplantation Research. *J Vis Exp* 2019.
24. , Guide for the Care and Use of Laboratory Animals, 9th edition. Washington (DC): 2011.
25. Sarhane KA, Tuffaha SH, Broyles JM, Ibrahim AE, Khalifian S, et al. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and molecular aspects, current challenges, and novel concepts. *Front Immunol* 2013;4:406.
26. Cendales LC, Kanitakis J, Schneeberger S, Burns C, Ruiz P, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. *Am. J Transplant* 2008;8:1396-1400.
27. Fleissig YY, Beare JE, LeBlanc AJ, Kaufman CL Evolution of the rat hind limb transplant as an experimental model of vascularized composite allotransplantation: Approaches and advantages. *SAGE Open Med* 2020;8:2050312120968721.
28. Meuli-Simmen C, Eiman T, Alpert BS, Meyer VE, Buncke GM, et al. Fibromuscular proliferation in finger arteries after hand replantation: a case report. *Microsurgery* 1996;17:551-554.
29. Griffin PA, Tan E, Katsaros J Late arterial thrombosis in a replanted thumb: a case report. *J Reconstr Microsurg* 1989;5:75-77.
30. Etra JW, Shores JT, Sander IB, Brandacher G, Lee WPA Trauma-induced Rejection in Vascularized Composite Allotransplantation. *Ann Surg* 2020;271:e113-e114.
31. Goutard M, Lellouch AG, Dussol B, Lantieri LA Facial Trauma 8 years after a Face Transplantation. *Plast Reconstr Surg Glob Open* 2021;9:e3575.

32. Kanitakis J, Petruzzo P, Gazarian A, Karayannopoulou G, Buron F, et al. Capillary Thrombosis in the Skin: A Pathologic Hallmark of Severe/Chronic Rejection of Human Vascularized Composite Tissue Allografts? Transplantation 2016;100:954-957.

Table 1

	Group 1 Syngeneic		Group 2 (Allo)		Group 3 (Allo)		Group 4 (Allo)	
	No Immunosuppression		Transient Immunosuppression		Suboptimal Immunosuppression		Standard Immunosuppression	
	Week 2	Terminal	Week 2	Terminal	Week 2	Terminal	Week 2	Terminal
Skin*	1.33±0.17	0.33±0.17	1.75±1.25	4.00±0.00	2.00±0.00	2.63±0.90	1.50±0.50	1.50±0.87
Muscle #		1.83±0.60		3.60±0.40		2.80±0.49		2.00±0.87
* Skin Biopsy Grade								
0	No signs of rejection							
1	Mild perivascular dermal cell infiltrate							
2	Diffuse dermal cell infiltrate, interface reaction, sporadic cell infiltration of epidermis							
3	Moderate to severe cell infiltration of epidermis, epidermal cell necrosis							
4	Severe cell infiltration, major epidermal necrosis, loss of epidermis							
# Muscle Biopsy Grade								
0	Normal muscle tissue							
1	Mild perivascular cell infiltrate							
2	Diffuse mononuclear cell infiltrate							
3	Moderate to heavy infiltrate, some disruption of tissue							
4	Heavy infiltrate, myocyte necrosis, tissue damage, fibrotic scarring							

Table 2

	First External Rejection Event			
	Group 1 Syngeneic	Group 2 (Allo)	Group 3 (Allo)	Group 4 (Allo)
	No Immunosuppression	Transient Immunosuppression	Suboptimal Immunosuppression	Standard Immunosuppression
Days Post Surgery	No events	19.4±2.87	39.8±3.57*	No events

Figure 1

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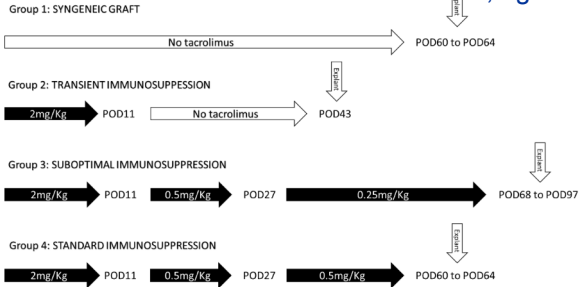


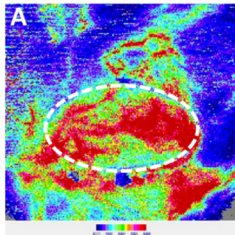
Figure 2
Fig. 2

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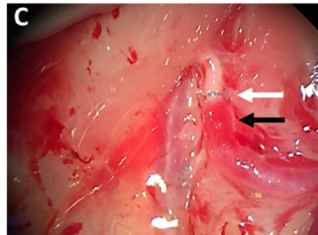
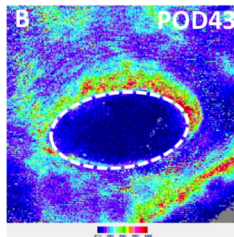


Group 2: Transient
Immunosuppression

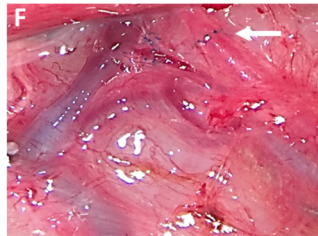
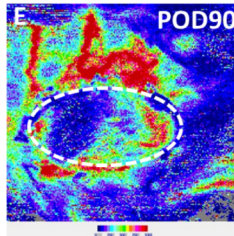
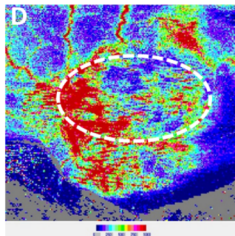
POD14



Experiment



Group 3: Suboptimal
Immunosuppression



Group 4: Standard
Immunosuppression

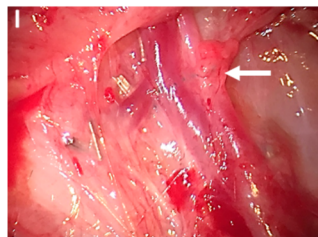
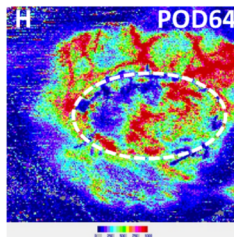
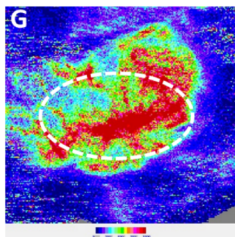


Figure 3

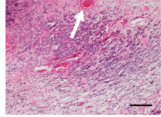
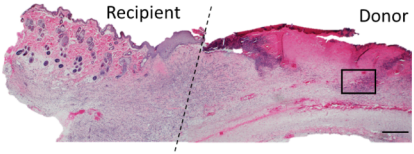
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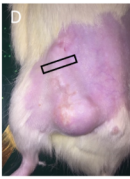
Group 2: Transient Immunosuppression



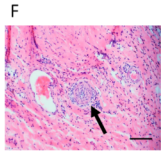
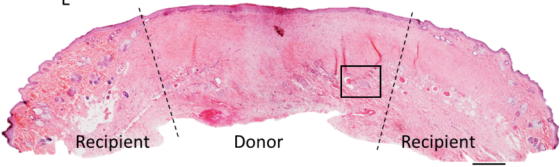
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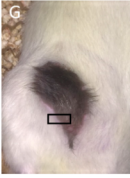
Group 3: Suboptimal Immunosuppression



E



Group 4: Standard Immunosuppression



H

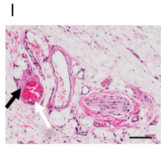
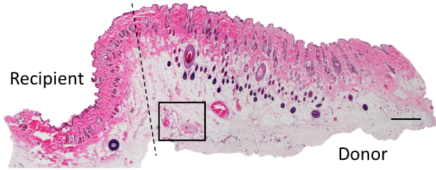
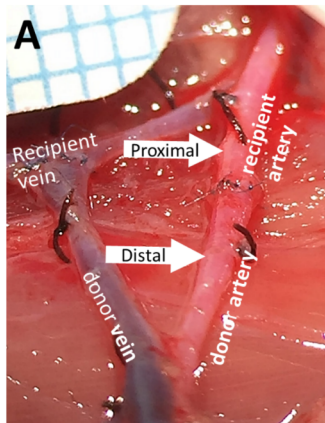
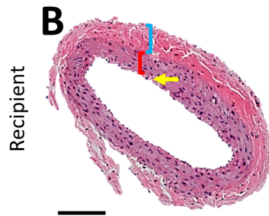


Figure 4
Fig. 4

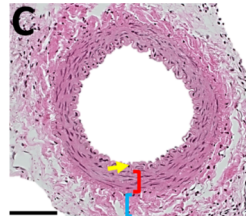
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Group 2: Transient
Immunosuppression



Group 3: Suboptimal
Immunosuppression



Group 4: Standard
Immunosuppression

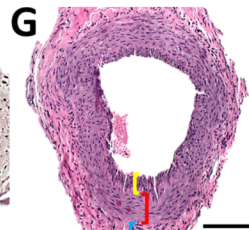
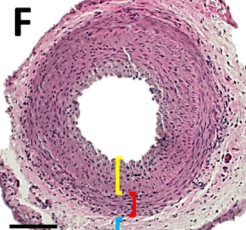
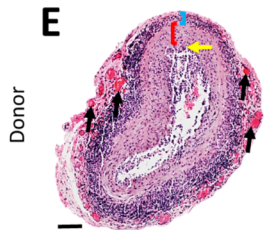
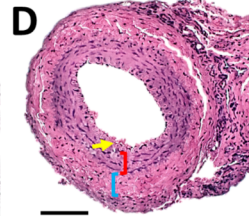


Figure 5

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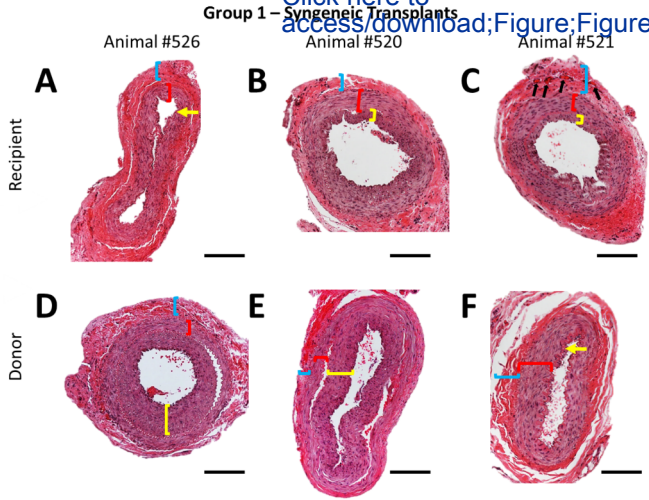


Figure 6

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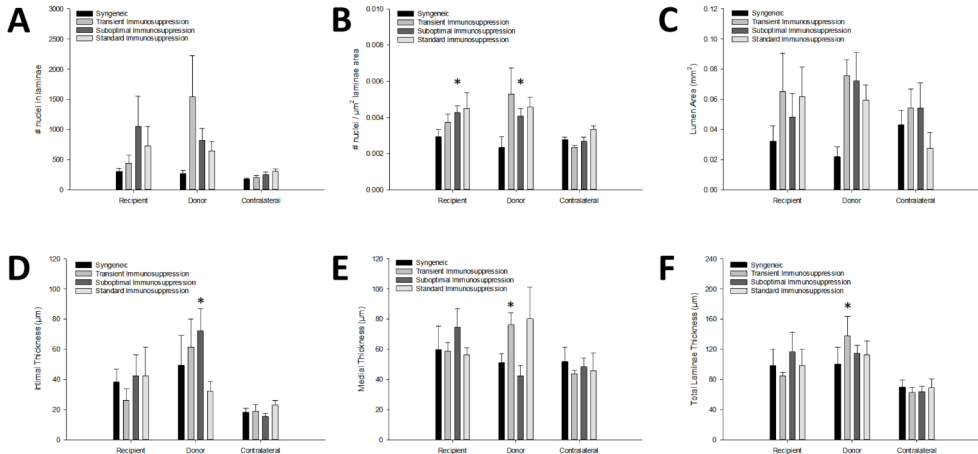
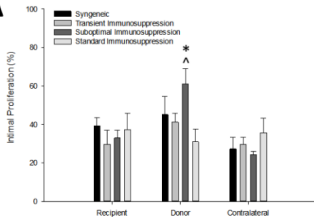


Figure 7

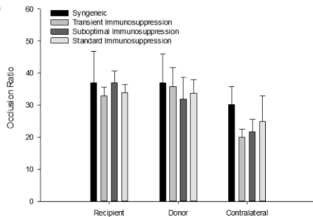
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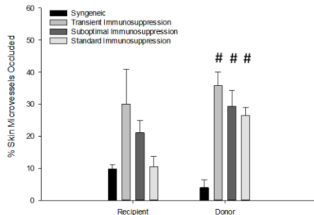
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C



D

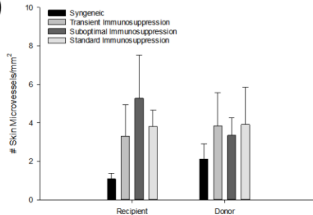
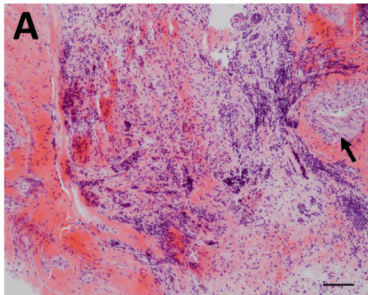


Figure 8

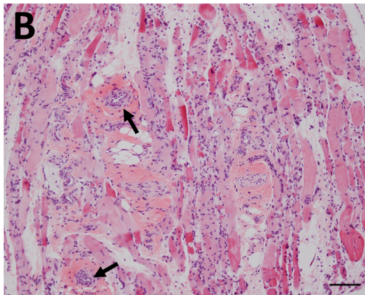
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Group 2: Transient
Immunosuppression



Group 3: Suboptimal
Immunosuppression



Group 4: Standard
Immunosuppression

