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

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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.

15. SUBJECT TERMS

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

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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	25%

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%

- **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 had technical issues with the rat surgery in Quarter 2, and these were resolved by quarter 3 and we were able to complete the goals in Major Task 2 of the SOW, having transplanted 4-5 animals in groups 1-4. As a reminder, Table 1 shows the animal strains and tacrolimus dose used for group 1-4. While tacrolimus is not needed to control rejection in a syngeneic Lewis to Lewis transplant model, in group 2 we have added the standard dose of tacrolimus which has been shown to maintain allogeneic flaps indefinitely. This group was included as we (and others) have noted that tacrolimus can be toxic, and result in weight loss and failure to thrive. Group 2 will allow us to determine if the presence of tacrolimus in the absence of alloreactivity has any effect on C3d deposition.

Table 1: **Rat models used in Groups 1-4**

Group	Strain	Tacrolimus dose
1	Lew > Lew	No tacrolimus
2	Lew > Lew	Standard (2mg > 0.5mg)
3	BN > Lew	Standard (2mg > 0.5mg)
4	BN > Lew	Suboptimal (2mg>0.25mg)

Figure 1 shows a representative Lewis animal from Group 3, who received an allogeneic flap from a BN donor rat, demonstrating a viable graft with good hair growth.

Figure 1: Animal 938 from group 3 (BN>Lew, standard immunosuppression), at 48 hours and week 1, 2, 3 and 4 post-transplant. Note that hair loss at week 3 and week 4 is due to shaving a small area to collect skin biopsies.

In addition, we are pleased to report that Mr. Max Malkani has done an excellent job of mounting and cutting tissue blocks from the various experimental groups and is working with Dr. Kelm and Mr. Beare on performing H&E staining of the tissue. In addition, we have successfully stained representative animals for C3d and other markers. These experiments will allow us to establish the baseline expression of C3d in both syngeneic and allogenic recipients. This in turn will allow us to assess the effect of CD21 blockade on C3d expression in subsequent groups.

We are pleased to report that the selected C3d and CD21 reagents work well in both the Lewis and BN animals. Group 1 and Group 2 are both syngeneic Lewis transplant controls. Not all of the tissue blocks are stained but initial results suggest that there is little C3d deposition in a Lewis OMC graft to a Lewis recipient at either week 1, week 2, or week 4. Note that group 1 animals do not receive any tacrolimus. Preliminary stains suggest that the addition of tacrolimus to the syngeneic model (Group 2) does not increase the level of C3d deposition in the skin.

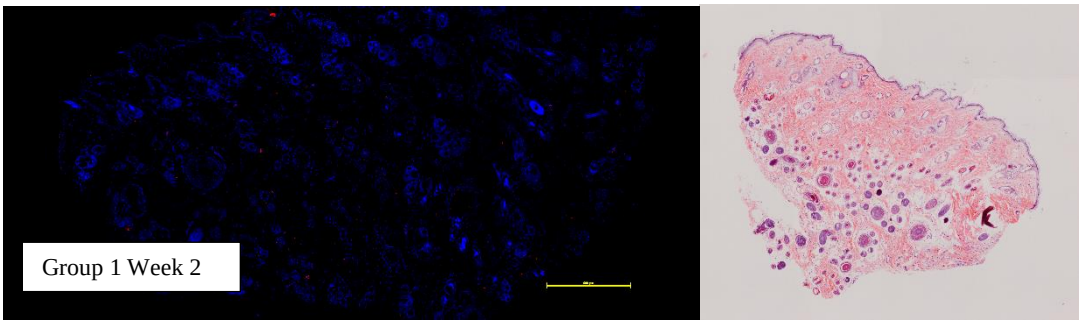


Figure 2: Skin biopsy of Lewis to Lewis Syn Tx (Group 1), Week 2. H&E stain is on the right, and on left is stain for DAPI (blue color) and C3d (red color). DAPI stains nuclear DNA. CD3d deposition is detected using a goat anti rat polyclonal antibody(R&D Systems; Cat#AF2655), with a secondary donkey anti-goat monoclonal antibody conjugated with Alex Fluor 568 (Abc am; Cat# ab175704). This sample at week 2 shows minimal C3d deposition, at a similar level seen in control C3d staining of normal Lewis skin.

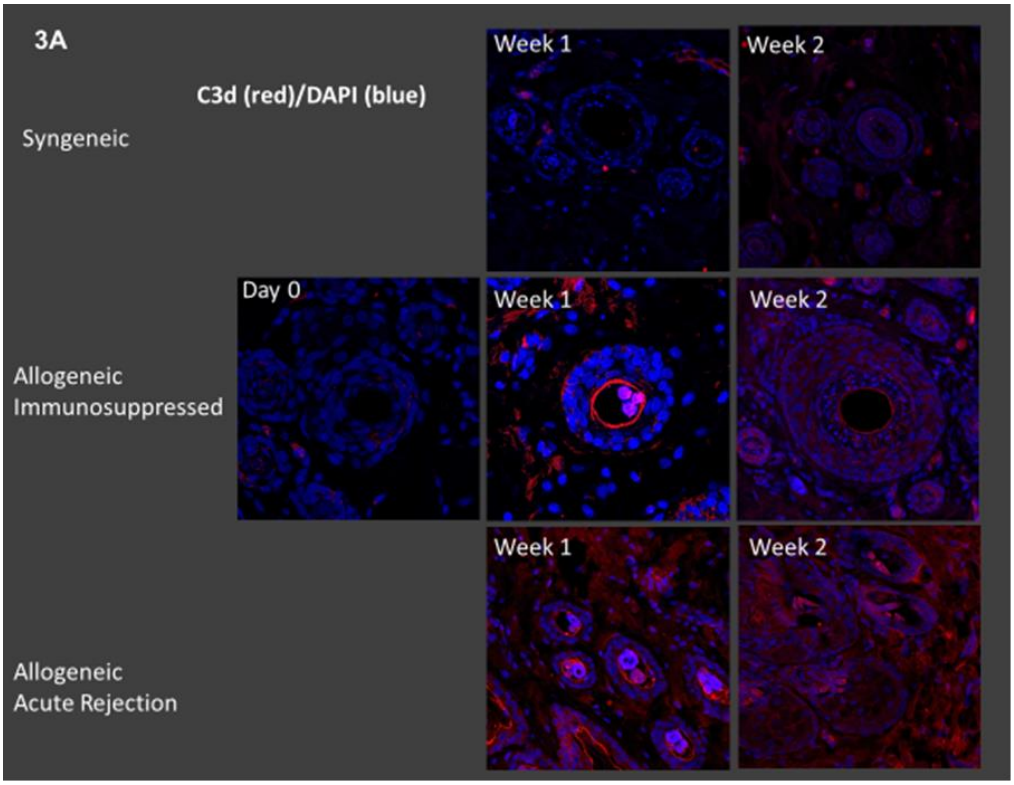


Figure 3: C3d expression (3A) and C4d expression (3B) in representative skin biopsies. Note the specific staining of hair follicles in week 1 (3A). In contrast C4d did not show specific staining of either week 1 or week 2 samples (3B).

In initial analyses, animals receiving an allogeneic graft have demonstrated localized C3d deposition, with more diffuse staining at week 2 post-transplant (Figure 3A). 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.

This finding is highlighted in Figure 3A, which shows the unique and strong C3d staining of rat hair follicles at week 1 in both the standard immunosuppression and accelerated rejection model. This staining was still present but less intense at week 2 in the standard immunosuppression group, and become more diffuse in the accelerated rejection model. While these standard immunosuppression animals do not demonstrate severe ACR, we know that mild ACR is ongoing, and animals show early signs of CR including small vessel vasculopathy, and mild vasculopathy of the femoral vein. (These observations were submitted in a manuscript to the Journal Transplantation, which is attached in the appendix, and is under review.) 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.

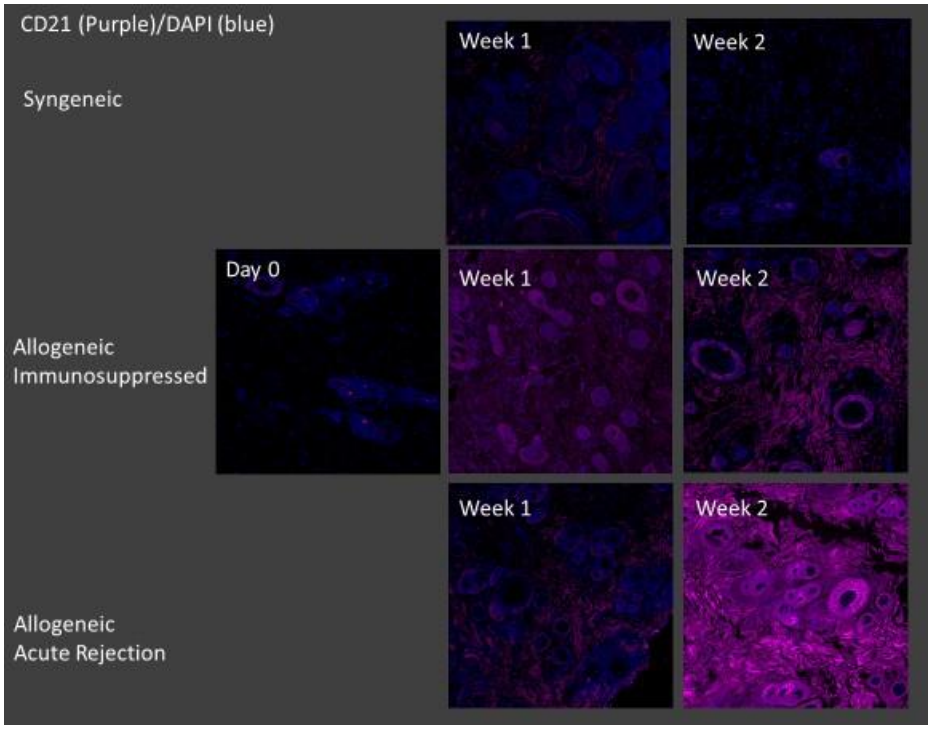


Figure 4: Staining of CD21 of skin biopsies Note intense staining of the acute rejection group at week 2 suggesting the presence of precursor and memory B cells.

We performed a similar analysis of CD21 staining which is shown in Figure 4. 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 in 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 300 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.

In addition to H&E analysis, C3d deposition, we will also characterize expression of C4d, CD21, CD20 (B cells) CD68 (macrophages) CD3 (T cells), rat IgG and IgM, and CD31 as a marker of endothelial cells. Mr. Max Malkani did a terrific job with the tissue blocks and staining, and an

abstract of his work this summer was accepted to Research!Louisville, which is an annual meeting hosted by the University of Louisville that offers students, residents and fellows an opportunity to present their work. We have attached a copy of Mr. Malkani's poster in the appendix. He presented preliminary data suggesting that C3d may be a marker of both cellular rejection as well as it's known association with antibody mediated rejection. As shown in figure 3, while C3d stained during early and ongoing ACR, C4d showed similar non-specific staining in both syngeneic (not shown) and allogeneic animals.

At the end of the third quarter in year 1, Dr. Kelm and Mr. Beare began performing the transplants for group 5, which is a syngeneic Lew > Lew OMC transplant, but with an additional two hours of cold ischemia time. In this model, the donor graft is harvested as normal, but then flushed with heparinized saline, wrapped in sterile guaze soaked in sterile saline, placed in a covered petri dish and placed at 4C for two hours. In first few animals, after the cold ischemia time, the graft was again flushed with heparinized saline. Unfortunately, the first two animals bled profusely, even from the sutures, and the grafts were non-viable after the first 72 hours. Dissection of the graft revealed clots around the anastomosis. The protocol for this group was revised to reduce the amount of heparin used in the flushes (from 30U/mL to 10U/mL), and the second flush after the two hours of cold ischemia is now just cold saline with no heparin. We expect these changes should reduce the bleeding and vascular compromise seen in the first two animals.

In addition, in the third and fourth quarter we worked on the C3d blockade experiments using the $CR2_2$-IgG1 hybridoma construct that was provided to us by our colleague Dr. Cascalho. 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 the 2nd quarterly report, 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 expect to have this antibody for use in blockade experiments during the 1st quarter of year 2 of the project.

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

We are pleased to report that two members of the research team, Dr. Natia Kelm and Mr. Max Malkani benefited from training or professional development during the last year. Mr. Max Malkani is an undergraduate student at the University of Kentucky. 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 drafted and submitted an abstract comparing C3d and C4d staining during acute cellular rejection to Research!Louisville. This is an annual conference sponsored by the University of Louisville to promote health related research. His abstract was accepted and will be presented at the conference on October 27th.

Dr. Natia Kelm is a key member of the research group and performs all of the recipient microsurgery for the project, with Mr. Beare assisting. In June of 2021 she presented an overview of the project and the results of inflammatory marker assays to the Transplant Research Working Group at the

University of Louisville. This is a monthly research seminar for anyone interested in transplantation, and is designed to promote interdisciplinary collaboration and a forum for clinical and basic scientists to work together. Dr. Kelm did an excellent job and continues to participate in the group and receive feedback on her work. In addition, as part of the troubleshooting of problems encountered in the rat surgeries (discussed below in the changes/problems section), Dr. Kelm and Dr. Kaufman participated in multiple online meetings with Dr. BC Oh and Dr. Gerald Brandacher. Dr. Brandacher has graciously invited Natia to come to the microsurgery lab at JHMI to see some of their surgical techniques. Dr. Kelm will visit their lab and spend the day on October 31, 2021.

- **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 just submitted a manuscript to the journal Transplantation on the development of vasculopathy and acute and chronic rejection of skin in patterns that mimic the presentation seen in our clinical hand transplant program. The submitted manuscript is attached in the appendix section of this report, and is entitled "A rat VCA heterotopic hind limb model of vasculopathy with and without skin changes in the presence of maintenance immunosuppression" and was assigned the manuscript number TPA-S-21-01929 by the journal.

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

In the next quarter we plan to focus on **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. We will perform additional surgeries focused on accruing more groups with additional cold ischemia time and compare C3d and CD21 as well as the other markers to characterize the impact on C3d staining. We expect to receive the CD21 dimer blocking antibody and will initiate blockade experiments as soon as it is received. In addition, we will continue to process and stain samples from animal groups already transplanted. We expect to have another manuscript in draft form by the end of Quarter 1 which will focus on C3d staining in the control groups. We will also continue to work with Dr. Cascalho and Dr. Platt on analysis of complement depletion after surgery and how that might be affected C3d deposition in these animals.

4. **IMPACT:**

- **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.

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

Information on the specificity of C3d on both cellular and antibody mediated rejection has the potential to impact all fields of transplantation, and potentially diagnosis and treatment of autoimmune disease as well.

- **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.

5. **CHANGES/PROBLEMS:**

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

There were no changes in the scope or direction of the research, but we have spent more time than expected on modifications of the model in response to graft losses when cold ischemia time was increased. We are currently evaluating surgical techniques that may include the epigastric artery and vein as well as the femoral artery and vein as well as changes in how the graft is perfused prior to implantation. As discussed above we are working with colleagues here in Louisville and with Dr. Brandacher's lab at JHMI to find solutions. Animal survival has increased and after Dr. Kelm visits Baltimore we will restart the cold ischemia experiments.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
- **Changes that had a significant impact on expenditures**

In the last quarter of year 1, we encountered significant early graft loss when groups with an additional 2 hours of cold ischemia time were transplanted. We consulted with microsurgery experts at the University of Louisville, and also reached out to Dr. Gerald Brandacher and Dr. BC Oh at the Johns Hopkins University Medical School. At their suggestion we are now also perfoming an anastomosis of the epigastric artery and vein (normally these are ligated when the donor graft is harvested). These vessels significantly increase the blood flow to the skin, and we believe will stop the early skin sloughing seen in first 48 hours in the animal groups included two hours of cold ischemia. We transplanted fewer than expected animals in quarter 4 as time was needed to work on this technical issue.

- **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

6. **PRODUCTS:**

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

Journal publications. Beare JE, Fleissig Y, Kelm NQ, Reed RM, LeBlanc AJ, Hoying JB and Kaufman CL. Rat VCA heterotopic hind limb model of vasculopathy with and without skin changes in the presence of maintenance immunosuppression. Submitted and under review at the journal Transplantation.

The submitted manuscript is attached in the appendix section. We have acknowledged the support of this award in the submission.

Other publications, conference papers, and presentations.

Poster Presentation at Research!Louisville –

C3d as a Marker of both Cellular and Antibody Mediated Graft Rejection. Max Malkani, Natia Q. Kelm, Jason E. Beare, Amanda LeBlanc, and Christina Kaufman – Presented 10/27/21 – pdf of poster as Attachment II.

Website(s) or other Internet site(s)

None to report

Technologies or techniques

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

  None to report

- **Other Products***:*

- In first year of the grant we have accumulated more than 300 tissue blocks from animals transplanted and have confocal and digitized light images of immunohistochemistry and H&E stained slides from ~190 tissue samples.

- In the first year of the grant we evaluated commercially available antibodies directed against rodent CD21 that could be used a blocking agent. While there were three available, the large quantities required per animal (between 4 and 8 mg each) were beyond the scope of the grant budget. We

were provided a cell line by Dr. Cascalho, and we contracted with Precision Antibody in Columbia Maryland to produce the CD21 dimer IgG antibody. We obtained quotes from multiple companies (most of which were also beyond the scope of the budget) and selected Precision Antibody based on the cost and quality assurance testing provided. The antibody is currently in production and we expect to have 0.5 to 1 gm of CD21 dimer IgG antibody within the next 5-6 weeks.

7. 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. Cascahlo 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: 4 months at 10% effort, 7 months at 20% effort and 1 month at 50% effort.
Contribution to Project: Mr. Beare is responsible for ordering animals and 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. Mr. Beare was originally on the grant for 10% effort, but as he worked much more than this, we recently requested and were approved (1/15/21) to increase his effort on the grant to 20%.

Name: Natia Kelm PhD
Project Role: Microsurgeon

Nearest person month worked: 12 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 and has optimized histologic staining for Ox27, allowing us to distinguish if cells within the grafts are of donor or recipient origin. She will participate in all aspects of performing studies for this project.

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 and Dr. Kelm with preparation for surgical procedures. Mr. Malkani has worked hard, and was genuinely interested in helping with any aspect of the project. We will do everything we can to encourage him to continue to participate as his undergraduate studies allow.

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

Grant W81XWH1520037 under PI Dr. Scott Frey ended in September of 2021. Dr. Kaufman was a subaward of this grant at a 10% effort.

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

We do not have a formal arrangement as partners, but in the last quarter of year 1, we have consulted frequently with Dr. Gerald Brandacher and Dr. BC Oh at the Johns Hopkins University Medical School on technical issues with the microsurgery in the cold ischemia groups, as mentioned in the above sections.

8. SPECIAL REPORTING REQUIREMENTS

An updated Quad Chart has been attached to this report

9. APPENDIX

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

Appendix II: A pdf of the poster presented at Research!Louisville is attached.

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

W81XWH-20-1-0943

PI: Christina L. Kaufman PhD **Org:** University of Louisville **Award Amount:** $500,000

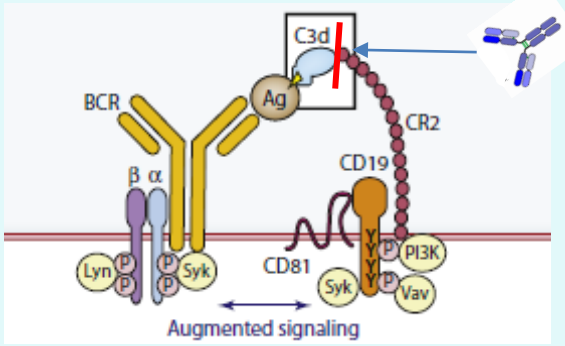
Study Aims

• **Specific Aim I**: Determine the level of C3d in syngeneic and allogeneic (BN to Lewis) osteomycutaneous (OMC) flap transplants, and the effect of C3d blockade and ischemia time on the incidence and severity of graft rejection in a model of acute rejection.

• **Specific Aim 2 -** Determine the lowest level of tacrolimus immunosuppression required to maintain OMC flaps free of rejection in allogeneic OMC flaps that receive C3d blockade on the day of transplant

Approach

Perform syngeneic (Lewis to Lewis) and allogeneic (BN to Lewis) rat OMC flap transplants and test how cold ischemia and/or C3d blockade changes pathology of the graft and the incidence and severity of rejection and vasculopathy



Anti CR2 should prevent C3d mediated augmentation of immune activation

Blockade of C3d binding to CR2 should prevent augmented signaling

Accomplishment: We have obtained reagents directed against CR2 (or CD21) that we hypothesize will block the augmented signaling induced by C3d, and reduce rejection in our experimental osteomyocutaneous flap model of VCA

Timeline and Cost

Activities	CY21	CY22
Perform OMC flap transplants		
Characterization of C3d levels and Staining (confirm higher in rejection)		
Determine if C3d blockade mitigates cold ischemia and reduces rejection		
Determine if C3d blockade at time of Tx reduces immunosuppression needed to control rejection		
Estimated Budget ($500,000)	**~$250K**	**~$250K**

CY21 Goals

☑ Achieve IACUC and ACURO study approval
☑ Train new personnel in microsurgery procedures
☑ Obtain reagents to identify and block C3d in the rat
☐ Confirm blockade of C3d reduces immune activation

CY22 Goals –

☐ Complete all planned experimental groups
☐ Determine if C3d Blockade reduces requirement for tacrolimus

Comments/Challenges/Issues/Concerns

• Reduced personnel costs are expected to be offset by increased costs for animals and production of CR2-IgG1 blockade reagent

Budget Expenditure to Date

Projected Expenditure: ~$250K
Actual Expenditure: ~$197K

Updated: October 27, 2021

Transplantation

A rat VCA heterotopic hind limb model of vasculopathy with and without skin changes in the presence of maintenance immunosuppression

--Manuscript Draft--

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Abstract:	Abstract Background Clinically, graft loss in vascularized composite allograft (VCA) recipients is more often related to vasculopathy and chronic rejection (CR) than acute cellular rejection (ACR). No single experimental VCA model has exhibited presentation of vasculopathy with or without skin involvement as seen clinically. We present a rat osteomyocutaneous (OMC) flap model using titrated tacrolimus administration that mimics graft rejection paradigms observed in our clinical hand transplant program. Methods Four groups were tested. Syngeneic controls, Allogeneic Acute Rejection (2mg/kg for 12 days), Allogeneic Suboptimal Immunosuppression (2mg/kg, then stepwise reduction to 0.5 and 0.25mg/kg at day 12 and 27), and Allogeneic Standard Immunosuppression (2mg/kg for 12 days, then reduce to 0.5mg/kg). Animals were monitored for ACR, vasculopathy and CR of the skin. Results Transient immunosuppression resulted in severe ACR within two weeks of stopping tacrolimus. Standard Immunosuppression resulted in minimal rejection, but subclinical signs of vasculopathy including capillary thromboses and lumen narrowing in arterioles in donor skin. Further reduction in tacrolimus led to frank femoral vasculopathy and CR of the skin. Conclusions: Titration of tacrolimus in allogeneic VCA model resulted in presentation of ACR and vasculopathy with and without CR of skin. This adjustable experimental model of VCA 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 recipients.

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A rat VCA heterotopic hind limb model of vasculopathy with and without skin changes in the presence of maintenance immunosuppression

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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 daily operations of the experiments. J.B., Y.F., and N.K. prepared tissue samples, performed histology staining, and imaged the tissue samples. J.B. performed histological measurements and statistical analyses of data. J.B., Y.F., R.R., N.K., A.L., J.H., and C.K. participated in writing of the manuscript.

Disclosures

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Abbreviations

ACR, acute cellular rejection

CR, chronic rejection

EEL, external elastic lamina

IEL, internal elastic lamina

MHC, major histocompatibility complex

OMC, osteomyocutaneous

POD, post-operative day

SOT, solid organ transplant

VCA, vascularized composite allograft

Abstract

Background Clinically, graft loss in vascularized composite allograft (VCA) recipients is more often related to vasculopathy and chronic rejection (CR) than acute cellular rejection (ACR). No single experimental VCA model has exhibited presentation of vasculopathy with or without skin involvement as seen clinically. We present a rat osteomyocutaneous (OMC) flap model using titrated tacrolimus administration that mimics graft rejection paradigms observed in our clinical hand transplant program.

Methods Four groups were tested. Syngeneic controls, Allogeneic Acute Rejection (2mg/kg for 12 days), Allogeneic Suboptimal Immunosuppression (2mg/kg, then stepwise reduction to 0.5 and 0.25mg/kg at day 12 and 27), and Allogeneic Standard Immunosuppression (2mg/kg for 12 days, then reduce to 0.5mg/kg). Animals were monitored for ACR, vasculopathy and CR of the skin.

Results Transient immunosuppression resulted in severe ACR within two weeks of stopping tacrolimus. Standard Immunosuppression resulted in minimal rejection, but subclinical signs of vasculopathy including capillary thromboses and lumen narrowing in arterioles in donor skin. Further reduction in tacrolimus led to frank femoral vasculopathy and CR of the skin.

Conclusions: Titration of tacrolimus in allogeneic VCA model resulted in presentation of ACR and vasculopathy with and without CR of skin. This adjustable experimental model of VCA 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 recipients.

Introduction

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

Allograft vasculopathy (AV) has been defined as a diffuse stenosis caused by concentric intimal hyperplasia and inadequate compensatory outward modeling[8]. Lesions are found only in the graft vessels, and the presentation that occurs months to years after transplant indicates a chronic vs. acute immune response. In graft vasculopathy observed in VCA recipients, all donor vessels appear to be affected in a fairly uniform manner involving progressive and diffuse intimal hyperplasia of arteries, although veins can also be affected[9,10]. In some cases, this vasculopathy progresses to vessel narrowing and eventually allograft ischemia[10-13]. While the terms vasculopathy and chronic rejection (CR) are sometimes used interchangeably in cardiac transplantation, VCA allografts have demonstrated multiple targets of CR including the vessels, skin and adnexal structures[14]. Patients have presented with 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[14].

There remains much speculation regarding vasculopathy pathogenesis; 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[5,10,11,14-17]. The common mechanisms and presentation of acute and severe vasculopathy and CR in 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 adaptable animal VCA models that will allow for 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 injury. In this study we utilized a modified rat heterotopic hind limb allograft[18] to develop an adaptable model of VCA which, with carefully titrated immunosuppression, allows for the study of ACR, CR, and vasculopathy as seen in a clinical setting.

Materials and Methods

Animals

Complete major histocompatibility complex (MHC) mismatched male Brown-Norway (BN, RT1.A^n) and Lewis (Lew, RT1.A^l) rats aged 12-16 weeks and weighing 275-350 grams were used as VCA donor 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 facilities for at least one week prior to surgery. Procedures were performed in accordance with the University of Louisville Institutional Animal Care and Use Committee (IACUC protocol #18198) and the NIH *Guide for the Care and Use of Laboratory Animals*[19].

Donor Surgery

Donor grafts were recovered for implantation as previously described[18]. Femoral vessels of donor BN (allograft) or Lew (syngeneic) animals were mobilized by ligating and cutting all vessels other than those providing arterial flow to and venous return from the graft, which consisted of tibia, fibula, surrounding muscles, nerve, and skin. Knee and ankle joints were disarticulated, and the artery and vein pedicle was cut proximally near the inguinal ligament. One ml of heparinized saline (30 Units/ml) was injected into the artery to flush the graft of blood. After graft recovery, the donor rat was euthanized via pneumothorax.

Recipient Surgery

Recipient animals were anesthetized using 2.5% isoflurane via endotracheal tube, then prepared for graft transplantation as previously described[18]. Donor vessels were anastomosed venous end-to-side, arterial end-to-end using 10-0 nylon interrupted sutures (Fig 1A). Visual assessment of anastomoses assured effective graft reperfusion. Total ischemic time was 60-90 minutes. The graft was inset into the inguinal pocket (Fig 1B) and oriented upside down, with ankle joint superior and knee joint inferior (Fig 1C).

Upon removal from anesthesia, 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 post-operative day (POD) 3.

Immunosuppressive Regimens

A schematic diagram of the four groups and dosing regimens is found in Fig 2. Syngeneic Lew recipients (n=3) did not receive immunosuppression throughout the experiment, emulating clinical replant recipients without alloreactivity; these animals were monitored daily and explanted on POD60-POD66. Allogeneic recipients were weighed twice weekly and administered daily subcutaneous injections of 2 mg/kg tacrolimus until POD11. In the Acute Rejection model, which was designed to produce early ACR with involvement of the skin, immunosuppression was discontinued after POD11; these subjects were monitored daily until POD41-POD44, when rejection necessitated explant (n=5). The 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). The 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. First clinical sign of rejection, including edema, maculopapular rash, erythema, and skin desquamation[2,20] were recorded. Macroscopic images of the grafted area were captured daily and scored by a double-blinded observer for rejection.

Laser Doppler Imaging

A Moor LDI2-IR laser Doppler scanner (Moor Instruments, Wilmington, DE, USA) with infrared 785 nm laser diode was used to assess blood perfusion through the graft skin at POD14 and day of explant. Perfusion through the skin is measured on a colorimetric scale from no flow (blue) to high flow (red)(see Fig. 3).

Tissue Biopsy and Explant Surgery

On POD14 and biweekly thereafter, recipient rats were anesthetized via 2.5% inhaled isoflurane, 2 mm skin biopsies obtained, and the wound closed with absorbable 4-0 suture. At 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 taken proximally (recipient side) and distally (donor side) to the anastomosis (Fig. 5A). All tissue samples were fixed in 10% formalin prior to paraffin embedding.

Histologic Evaluation

For morphometry, 6 µm 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, sebaceous glands). Skin and muscle biopsies were graded by a blinded observer using criteria in Table 1.

Evaluation of Graft Vasculopathy

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

Results

Operative results

Donor operative time was approximately 45 minutes, while recipient operative time was approximately 1.5 to 2 hours. On rare occasions, minor post-operative 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, one animal in the Standard Immunosuppression group was found to have numerous masses in the inguinal cavity, peritoneal cavity, and on the 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 entirety of the study. On explant, macroscopic examination of the femoral artery revealed no signs of trauma or hypertrophy on either side of the anastomosis site (data not shown).

Acute Rejection animals exhibited sufficient perfusion to the graft on POD14, including robust recipient collateral feeding vessels (Fig. 3A); blood perfusion to the skin was completely ablated on the day of explant (POD43)(Fig. 3B). Surgical exposure of the anastomoses during explant revealed healthy recipient artery, while the donor artery distal to the anastomosis was severely inflamed and hypertrophied (Fig. 3C).

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

In Standard Immunosuppression animals, blood perfusion to the skin appeared unchanged from POD14 (Fig. 3G) to POD64 (Fig. 3H), indicating proper blood flow throughout the course of the study. Exposure of the anastomoses at explant revealed no outward signs of vascular trauma or thromboses (Fig. 3I).

Clinical Signs of Rejection and Skin Histopathology

Syngeneic recipients exhibited normal healing, healthy skin components, and proper donor hair growth throughout the study. No clinical signs of rejection were observed. Biopsies from POD14 as well as at time of explant (POD60-POD66) showed normal skin adnexal components with ACR score of Grade 0 or 1 (Table 1).

In the ACR animals, skin showed normal wound healing and donor hair growth during the first 3 weeks post-surgery, indicating proper vascularization to the flap. Identification of donor skin vs. recipient skin was readily apparent due to the color and growth direction of the hair. The first signs of rejection including skin redness, swelling and partial loss of hair were observed at POD19.4 $\pm$ 2.87 days (Table 2); by the time of explant, donor skin was necrotic and graft failure was evident in all Acute Rejection animals (Fig. 4A). 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). At explant, donor skin lacked adnexal structures, and epidermis was necrotic and atrophic (Fig. 4B). Microvessel thrombosis (Fig. 4C, white arrow) could be seen in donor skin, as well as extensive mononuclear cell infiltration. Muscle histopathology from explant revealed robust cellular infiltration, lack of cross striation and hypochromagenicity of muscle, and arteriole hyperplasia (black arrow, Supplemental Fig. S1A)(Table1).

In animals receiving Suboptimal Immunosuppression, clinical follow up showed normal VCA skin appearance with healthy hair growth at POD14. Identification of the donor vs. recipient skin was readily apparent. Skin biopsies at POD14 showed normal morphology with diffuse mononuclear cell infiltrate (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 POD39.8 $\pm$ 3.57 days (Table 2). Donor skin over the flap gradually reduced in size over the course of the experiment and was eventually covered by new epidermis (Fig. 4D). At explant, mononuclear cell infiltration was apparent in the donor skin (Fig. 4E), but to a lesser extent than that seen in ACR animals. Histopathology revealed a lack of adnexal structures in donor skin, with newly-formed epidermis covering the graft. Recipient skin exhibited healthy adnexal structure, epidermis, and vessel components. At greater magnification, arteriole wall thickening with perivascular infiltration (Fig. 4F, black arrow) was observed in the donor area of the skin. Muscle biopsy from time of explant revealed moderate cellular infiltration and decreased lumen diameter in muscle arterioles (Supplemental Fig. S1B, black arrows)(Table 1).

In the Standard Immunosuppression group, clinical follow up revealed normal VCA skin and hair throughout the entirety of the study (Fig. 4G). When immunosuppression was maintained at 0.5 mg/kg, recipient animals did not exhibit gross signs of rejection. One animal in this group did show some redness and swelling on POD44. However, this animal developed tumors throughout the graft and in the peritoneal cavity and lungs, necessitating 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 graft border at explant revealed that donor skin resembled recipient skin; regardless of source (donor or recipient), skin exhibited normal epidermis and adnexa without morphological change, and mild mononuclear infiltration (Fig. 4H). At higher magnification, microvascular thrombosis (white arrow) and vessel wall thickening (black arrow) were seen in the donor skin (Fig. 4I). Histology of explant muscle samples revealed mild cell infiltration and healthy microvessels (Supplemental Fig. S1C, white arrow)(Table 1).

Vessel Histopathology and Quantitative Measurement

Samples of the femoral artery proximal (recipient) and distal (donor) to the anastomosis were obtained as shown (Fig. 5A). Histological analyses of 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+medial laminae of the vessel. Donor lumen area was slightly but not significantly smaller (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 revealed in intimal thickness (Fig. 6D),

medial thickness (Fig. 6E), total laminae thickness (Fig. 6F), intimal proliferation (Fig. 7A), occlusion ratio (Fig. 7B), percentage of skin microvessels occluded (Fig. 7C), or skin microvessels/mm^2 (Fig. 7D).

In the ACR group, histology of the femoral artery proximal and distal to the anastomosis revealed robust mononuclear cell invasion in the medial layer of the donor artery (Fig. 5E) compared to recipient artery (Fig. 5B). An apoptotic layer of cells lining the lumen of the distal artery sample was observed (Fig. 5E), with pronounced microvascular thromboses in the adventitial layer (Fig. 5E, examples shown with black arrows). Severe vasculitis of the donor artery in this group required imaging this vessel sample at a lower magnification (10x); all other vessel images in Fig.5 were acquired with 20x magnification. Quantitative vessel measurements revealed increased albeit not significantly more total cells (Fig. 6A) and cells/ μm^2 (Fig. 6B) in the intimal+medial laminae. Lumen area (Fig. 6C) and intimal thickness (Fig. 6D) were unchanged, with increased medial thickness in the donor artery distal to anastomosis compared to contralateral vessel samples (One-way ANOVA,DF=2, F=8.081, p=.006, Holm-Sidak *post hoc* p=.005)(Fig. 6E). Similarly, total laminae thickness (intimal+medial layers) exhibited significant outward remodeling in donor artery compared to contralateral control vessel (One-way ANOVA, DF=2, F=6.090, p=.015, Holm-Sidak *post hoc* p=.016); donor artery approached significance compared to recipient artery (p=.065)(Fig. 6F). Despite a distinct change in lumen area in the donor artery of ACR animals compared to Syngeneic animals, this difference was not significant (Fig. 6C). Intimal proliferation and occlusion ratio 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). Skin microvessels/mm^2 were unchanged (Fig. 7D)

For Suboptimal Immunosuppression animals, vessel samples from the recipient artery exhibited healthy intimal and medial layers (Fig. 5C). In the donor artery, marked intimal hyperplasia was apparent (Fig. 5F, yellow bracket), and apoptotic cells lined the innermost layer of the intima along the lumen. Significantly more infiltrating cells/μm^2 of intimal+medial area were found (One-way ANOVA, DF=2, F=6.092, p=.015) both in the recipient (p=.023) and donor artery (p=.031) compared to contralateral (Fig. 6B). Lumen area was unchanged (Fig. 6C), while intima was significantly thicker in the donor vessel compared to contralateral control artery (One-way ANOVA, DF=2, F=5.747, p=.018, Holm-Sidak *post hoc* p=.016)(Fig. 6D). Medial thickness and total laminae thickness were unchanged (Fig. 6E and 6F, respectively). Intimal proliferation was significantly higher in donor vessel (One-way ANOVA, DF=2, F=13.310, p<.001) compared to both recipient (p=.005) and contralateral artery (p=.001)(Fig. 7A). Percentage of skin microvessels occluded was significantly higher in the donor skin compared to Syngeneic donor skin (One-way ANOVA, DF=3, F=9.785, p=.001, Holm-Sidak *post hoc* p=.004)(Fig. 7C). Skin microvessels/mm^2 were unchanged (Fig. 7D).

As in both insufficiently immunosuppressed groups, animals in the Standard Immunosuppression group exhibited normal vessel components on the recipient side of the anastomosis (Fig. 5D). Distally, intimal hyperplasia could clearly be seen in donor vessel (Fig. 5G), with a layer of apoptotic cells lining the innermost layer of the intima. All vessel characteristics measured were unchanged in recipient and donor artery samples compared to contralateral vessels (Fig. 6A-F, 7A-C). A robust increase in medial

thickness in the donor artery proved to be non-significant (Fig. 6E). Percentage of skin vessels occluded on donor skin of Standard Immunosuppression animals was significantly higher compared to Syngeneic donor skin (One-way ANOVA, DF=3, F=9.785, p=.001, Holm-Sidak *post hoc* p=.008)(Fig. 7C). Skin microvessels/mm^2 were unchanged (Fig. 7D).

Discussion

Dozens of experimental models of VCA have been described, each with their own advantages and drawbacks[21]. The model presented here was developed previously in our center[18], and offers key components that mimic complications observed in the clinic. The groups presented here were successful in modeling ACR without vasculopathy, vasculopathy without significant ACR or chronic skin rejection, and evidence of both chronic skin changes and vasculopathy. What still remains to be developed is a model of development of chronic skin changes in the absence of significant vasculopathy which has also been observed in VCA patients[14].

Our results can be interpreted by outlining each of the groups on a 'rejection spectrum,' with outwardly healthy vasculopathy animals and fully rejecting animals on opposite sides. This spectrum is influenced by two variables, the immunosuppressive dose and the duration of treatment. In the ACR group, when immunosuppression was halted 12 days post-operatively, clinically relevant rejection events followed in less than two weeks, a short time frame that did not allow vasculopathy to develop. In the Suboptimal Immunosuppression group, tacrolimus administration was extended but reduced in a stepwise fashion and maintained at lower doses until terminal time points;

this allowed both chronic skin rejection with loss of adnexal structures as well as vasculopathy to develop. In the Standard Immunosuppression group, tacrolimus dose was lowered on POD12 but maintained at a dose sufficient to keep the graft outwardly healthy. On the surface, skin and hair appeared normal throughout the course of the study, and blood perfusion was uniform throughout the flap at explant. However, closer analyses uncovered vascular pathology: narrowing of microvessel lumen in the skin, microvessel thromboses, and mild medial hyperplasia of the donor femoral artery distal to the anastomosis. Thus, the vascular injury present under the surface of the seemingly quiescent transplanted tissue may represent the beginning of a cascade of events that ultimately lead to graft vasculopathy; this places these animals on the low end of the rejection spectrum and most similar to the clinic, where the target is the lowest amount of immunosuppression which will keep the graft healthy. Importantly, this models the development of vasculopathy without significant skin rejection, which we have seen clinically in hand transplant recipients.

In the clinic, the driving forces behind the variable movement of individual patients along the rejection spectrum are still unknown. Cell-mediated rejection[2,22-29], donor-specific antibodies and HLA cross-reactivity leading to antibody-mediated rejection[11,23,24,29-36], and graft vasculopathy[10,15,23,24,37-40] – brought on via improper immunosuppression, accumulation of acute rejection events, and/or mechanical injury – have all been linked to VCA failure. While intimal hyperplasia is also influenced by other factors, myointimal proliferation and gradual luminal narrowing in the graft can be harbingers of graft ischemia, necrosis, and eventual failure of the VCA[24].

In our Standard Immunosuppression-induced vasculopathy model, it appears as though the vascular injury in the graft begins in the small vessels. Microvessels in the donor skin exhibited thromboses and lumen narrowing, while donor artery distal to anastomosis showed minimal signs of vasculopathy. This observation may point to a cascade of vascular involvement in rejection that begins in the smallest vessels, although further study is required to confirm this theory. Strengthening this argument, Kanitakis et al. highlighted a case in which capillary thrombosis presaged a larger vasculopathy that ultimately led to the loss of two grafted fingers in a human VCA patient[11]. Others have theorized that proliferation of smooth muscle cells and deterioration of the endothelial cell layer are significantly involved in the development of graft vasculopathy and ultimately CR[15]. In addition, the buildup of apoptotic cells near the vessel lumen plays a crucial role in intimal hyperplasia[39] and suboptimal perfusion to the graft, increasing the likelihood of ischemia and graft failure[15].

In summary, we present a reliable, reproducible tool for studying the underlying mechanisms involved in VCA graft vasculopathy and CR. 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. Anatomical representation of heterotopic osteomyocutaneous VCA model. A) Completed femoral vessel anastomoses between donor and recipient. The arteries are connected via end-to-end anastomosis while the veins are connected via end-to-side anastomosis to conserve native limb vasculature and drainage. B) Upon completion of anastomoses, the inguinal pocket is prepared for orientation of the graft. C) X-ray indicating the orientation of the graft, with the distal end of the graft positioned proximally in the inguinal pocket.

Figure 2. Schematic diagram of the tacrolimus dosing regimens utilized to produce each experimental group.

Figure 3. Laser Doppler imaging of a representative animal from each experimental group, with the border of the skin window marked by white dashed lines. In Acute Rejection, 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, 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, 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 4. Skin histopathology. At time of explant, Acute Rejection 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). Suboptimal Immunosuppression rejection animals exhibited pervasive hair loss and reduction in donor skin component area (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 5. Femoral artery histopathology. Samples of the recipient femoral artery and donor femoral artery were obtained proximal and distal to the anastomosis site, respectively (A). Regardless of group, 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 thromboses 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). 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 μm for all panels; images were captured at 20x magnification for all panels except E, which was captured at 10x magnification.

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. Acute Rejection 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.

Supplemental Figure S1. Representative images of muscle biopsy at time of explant for each group. Animals in the Acute Rejection group exhibited robust mononuclear cell infiltration, as well as hyperplasia of capillary vascular structures (A, black arrow). Suboptimal Immunosuppression animals showed moderate cell infiltrate, combined with compromised lumen diameter in muscle capillaries (B, black arrows). Standard Immunosuppression resulted in minimal cell infiltration, healthy muscle, and normal capillaries (C, white arrow). Scale bar = 100 µm.

Table 1. Rejection scoring for skin and muscle biopsies. Regardless of group, all samples taken at POD14 exhibited mild cell infiltration. At the time of explant, cell infiltration was resolved in Syngeneic animals, severe with major epidermal necrosis in ACR animals, moderate with some epidermal loss in CR animals, and mild to moderate in Standard

Immunosuppression animals. Muscle biopsies at explant revealed mild cell infiltrate in Syngeneic animals, heavy mononuclear cell infiltration with myocyte necrosis in ACR animals, moderate cell infiltrate in CR animals, and diffuse cell infiltrate in 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 CR animals compared to ACR animals (*p=.002).

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Table 1

	Syngeneic		Allogenic					
			Acute		Suboptimal		Standard	
	Week 2	Terminal	Week 2	Terminal	Week 2	Terminal	Week 2	Terminal
Skin*	1.16±0.44	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			
	Syngeneic	Allogenic		
		Acute	Suboptimal	Standard
Days Post Surgery	No events	19.4±2.87	39.8±3.57*	No events

Fig. 1

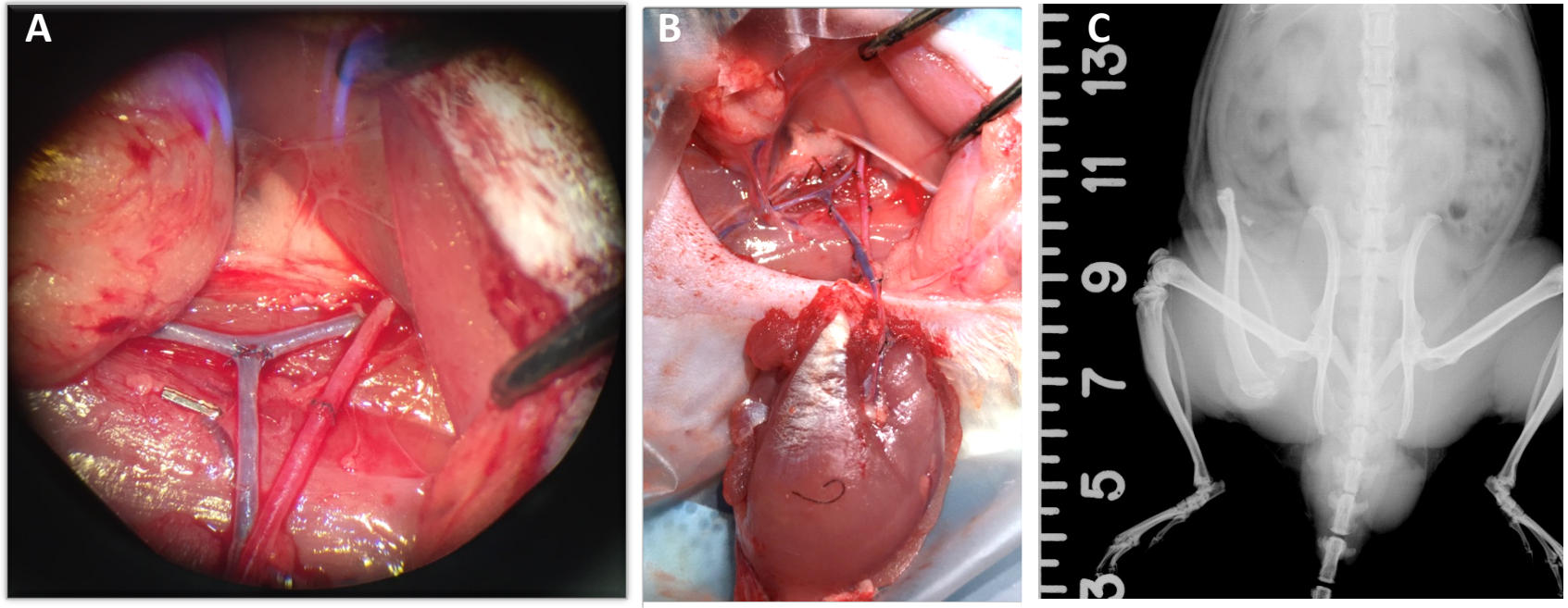


Fig. 2

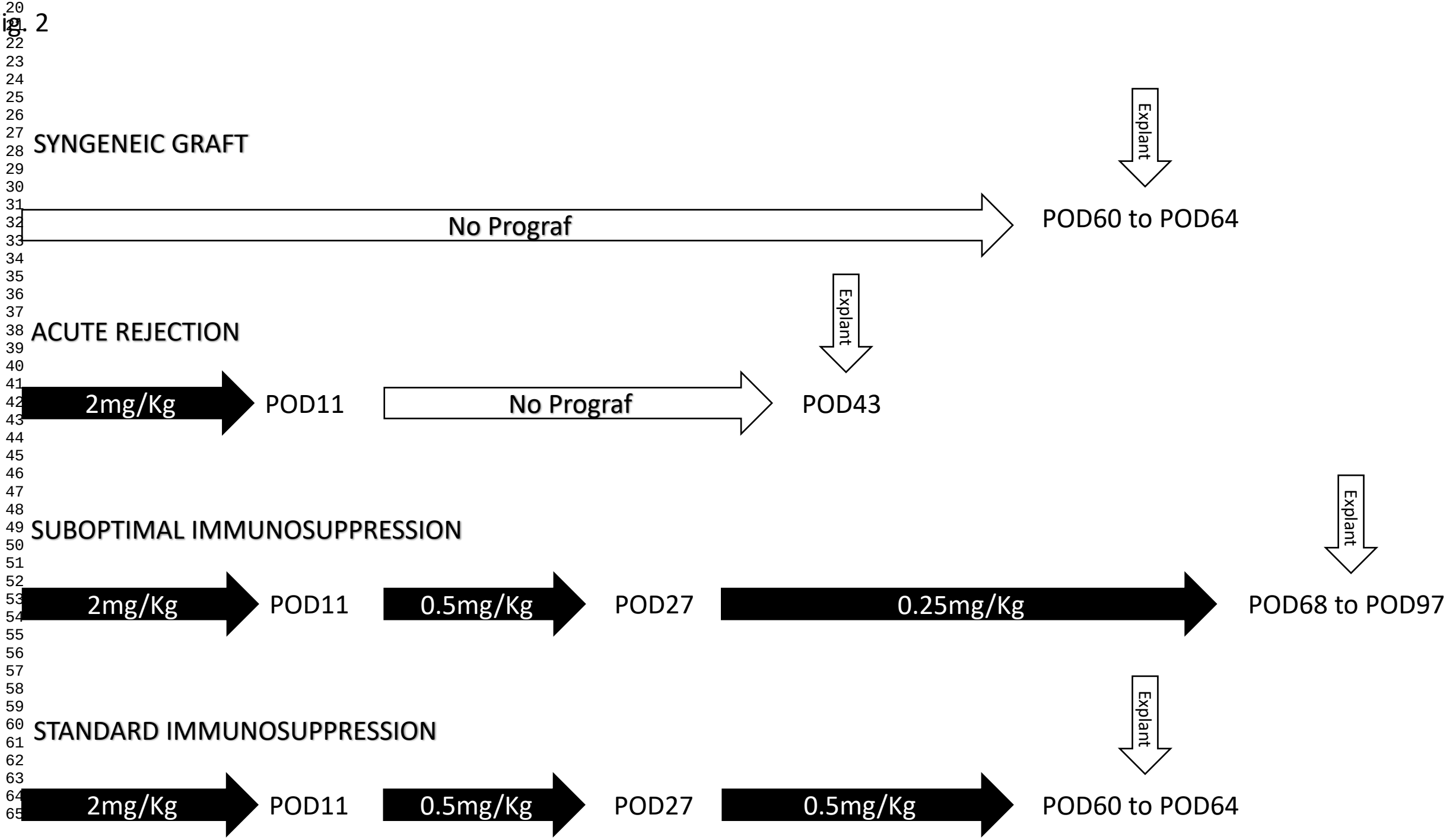

SYNGENEIC GRAFT
No Prograf
Explant
POD60 to POD64
ACUTE REJECTION
2mg/Kg
POD11
No Prograf
Explant
POD43
SUBOPTIMAL IMMUNOSUPPRESSION
2mg/Kg
POD11
0.5mg/Kg
POD27
0.25mg/Kg
Explant
POD68 to POD97
STANDARD IMMUNOSUPPRESSION
2mg/Kg
POD11
0.5mg/Kg
POD27
0.5mg/Kg
Explant
POD60 to POD64

Fig. 3

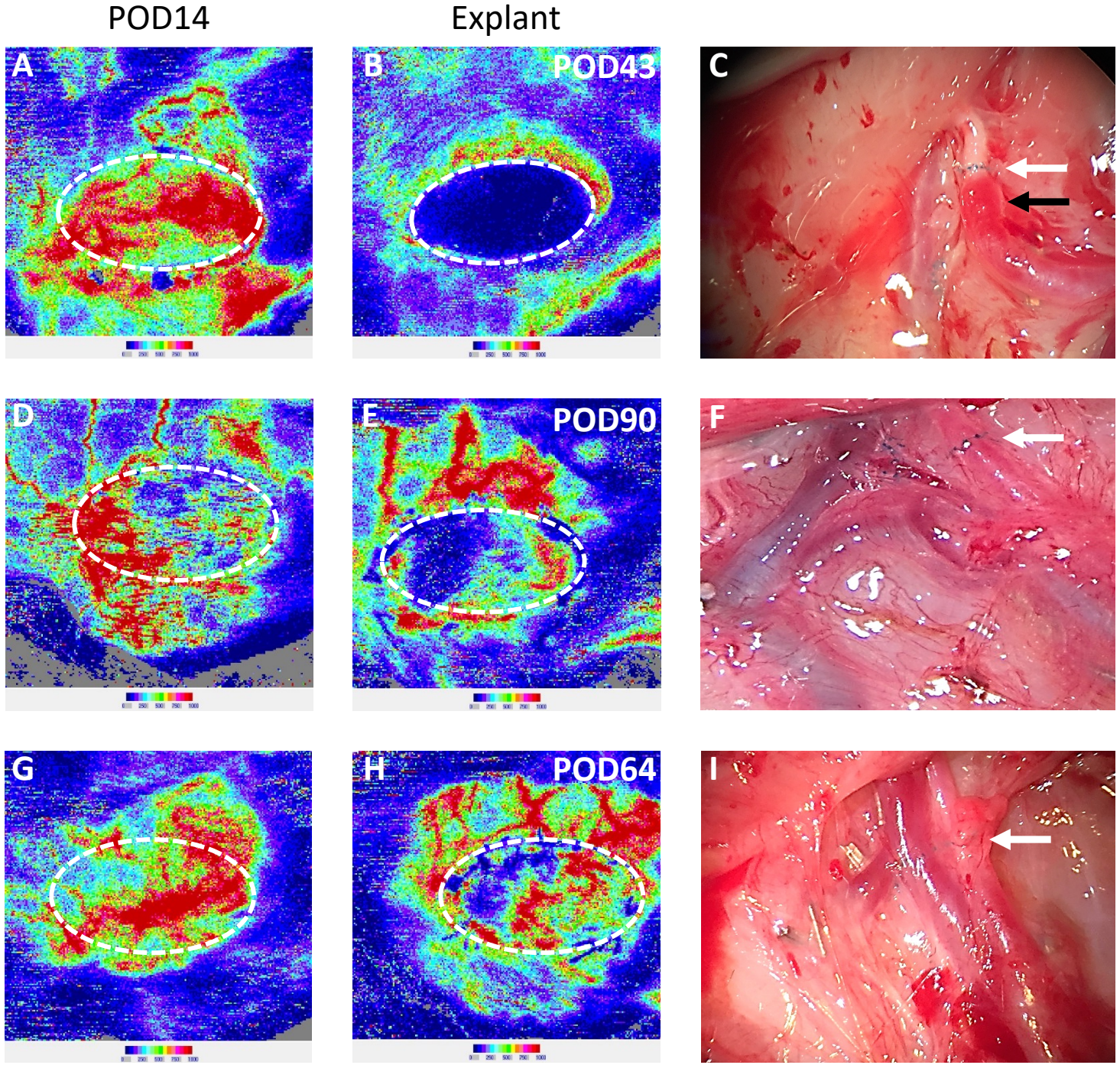

POD14
Explant
Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
A
B
POD43
C
D
E
POD90
F
G
H
POD64
I

Fig. 4

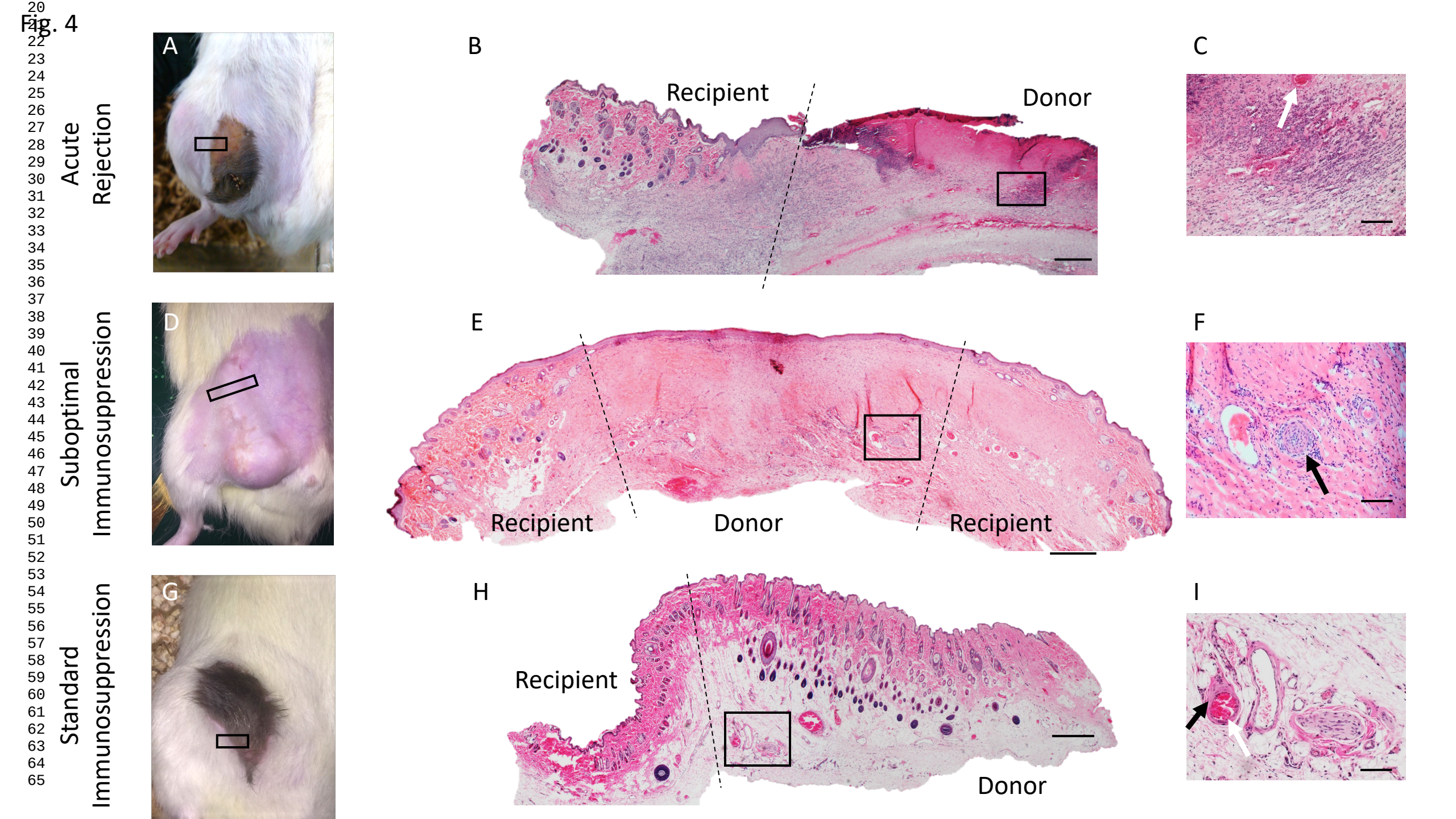
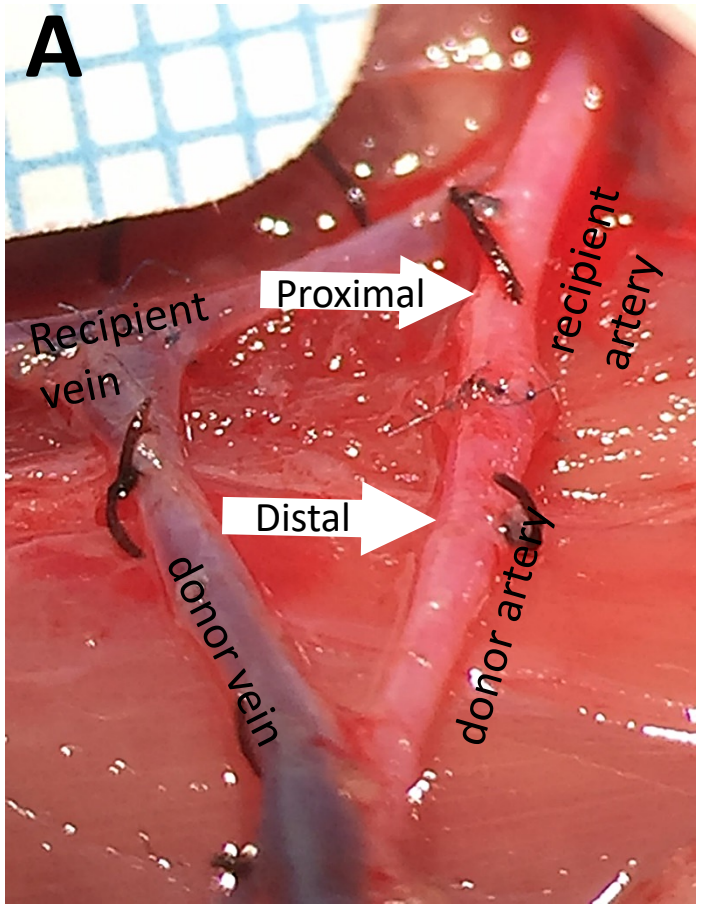

Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
A
B
C
D
E
F
G
H
I
Recipient
Donor
Recipient
Donor
Recipient
Recipient
Donor

Fig. 5


A
Proximal
Distal
Recipient vein
recipient artery
donor vein
donor artery

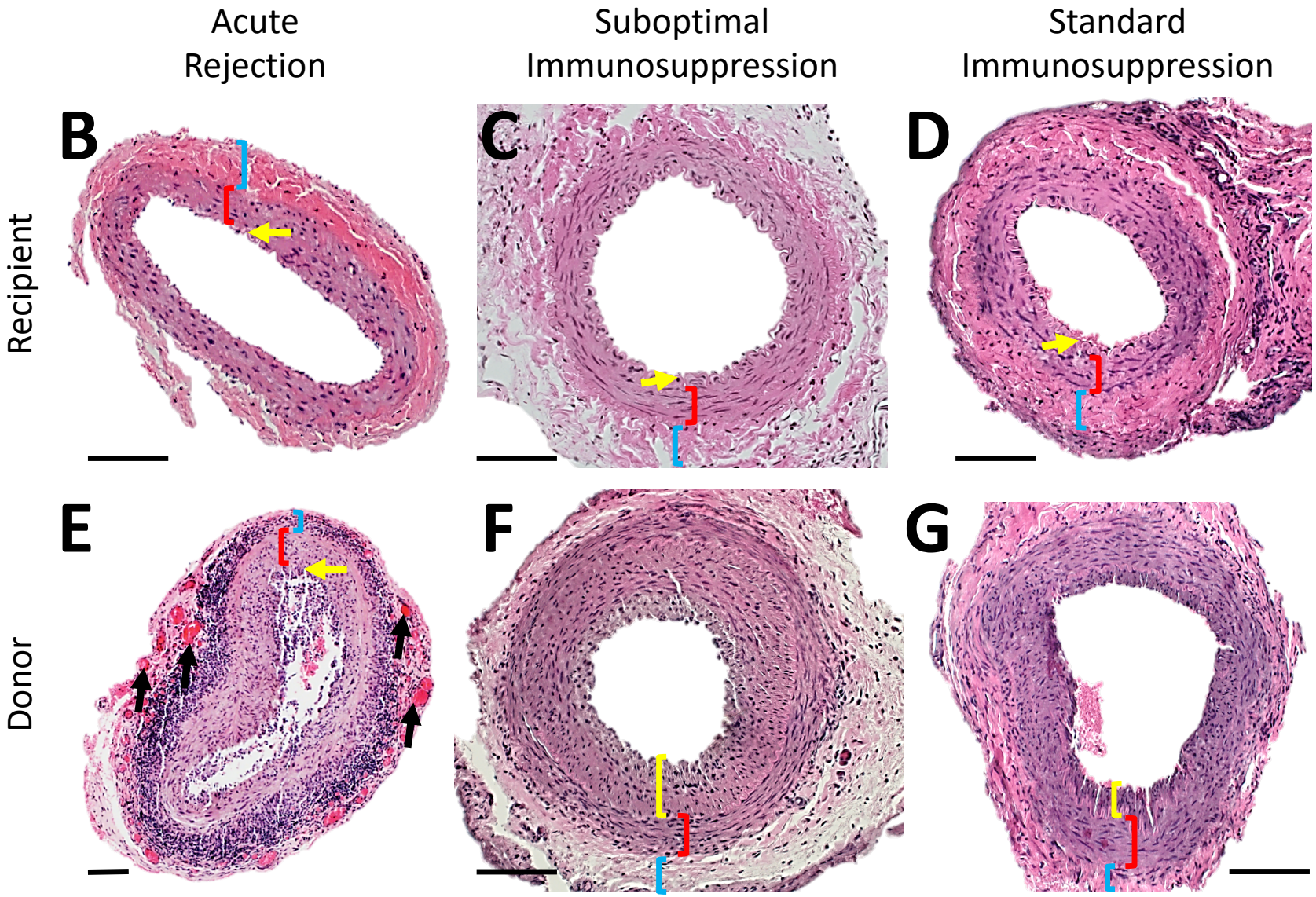

Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
Recipient
Donor
B
C
D
E
F
G

Fig. 6

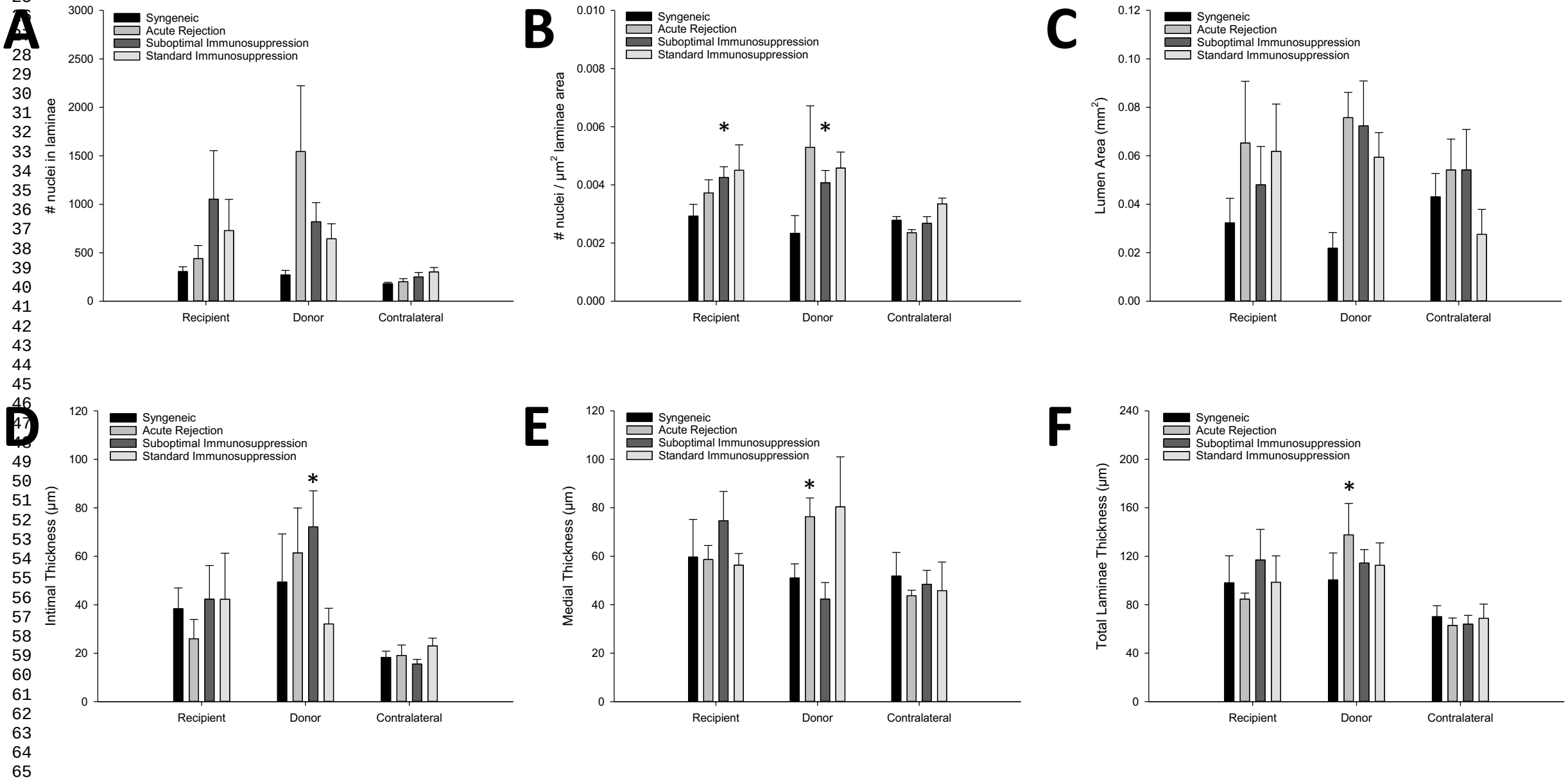
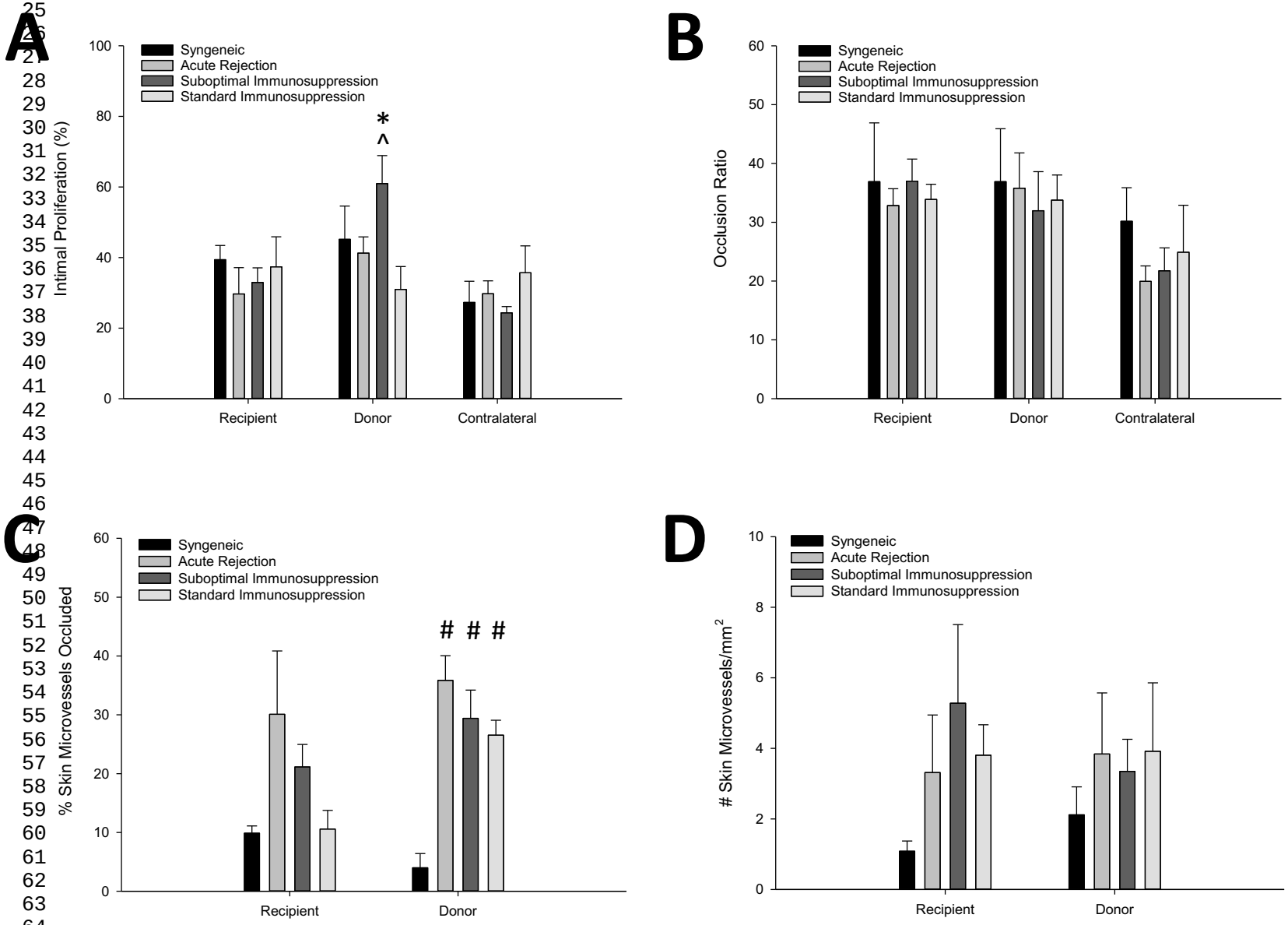
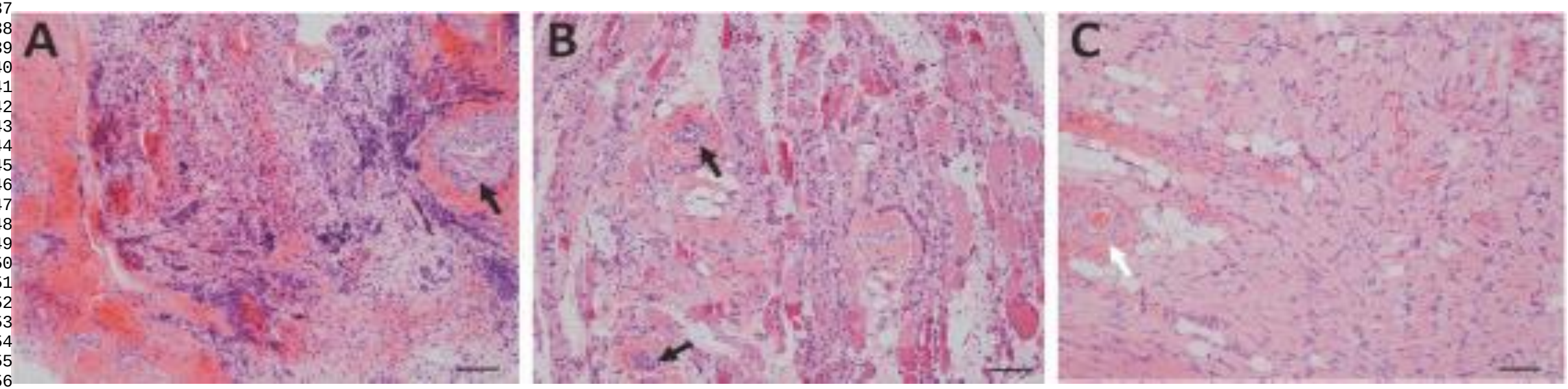

A
B
C
D
E
F
Syngeneic
Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
nuclei in laminae
nuclei / µm² laminae area
Lumen Area (mm²)
Intimal Thickness (µm)
Medial Thickness (µm)
Total Laminae Thickness (µm)
Recipient
Donor
Contralateral
*

Fig. 7


A
Syngeneic
Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
Intimal Proliferation (%)
100
80
60
40
20
0
*
^
Recipient
Donor
Contralateral
B
Syngeneic
Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
Occlusion Ratio
60
50
40
30
20
10
0
Recipient
Donor
Contralateral
C
Syngeneic
Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
% Skin Microvessels Occluded
60
50
40
30
20
10
0
# # #
Recipient
Donor
D
Syngeneic
Acute Rejection
Suboptimal Immunosuppression
Standard Immunosuppression
Skin Microvessels/mm2
10
8
6
4
2
0
Recipient
Donor

Simple Submission SDC file

Figure S1

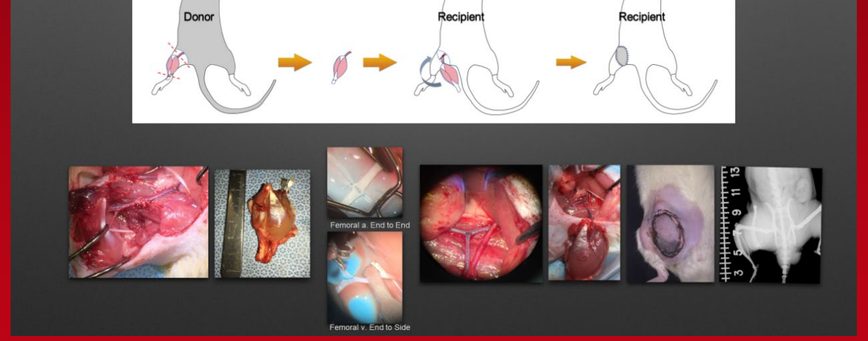


extremity or face. The University of Louisville VCA Program transplanted the first five hand transplants in the United States and the first hand transplant recipient in Louisville is now 22 years post transplant and doing well.

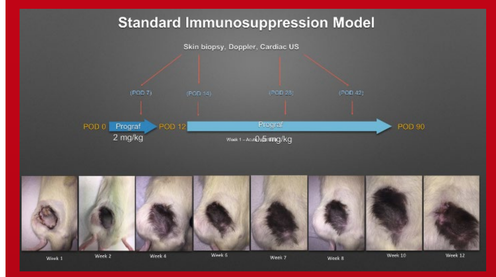
Like other types of transplants such as heart and kidney, VCA recipients have both cellular mediated rejection (primarily T and B cells) and humoral or antibody mediated rejection. C4d is the standard biomarker for analyzing antibody mediated rejection in transplanted allografts. C4d is a molecule that is cleaved when complement is activated (for example by antibodies binding to the graft) which then binds covalently to nearby tissue, thus serving as a marker for antibody mediated rejection. In vascularized composite allotransplantation (VCA), this marker is less useful as it can stain non-specifically in the skin. Additionally, deposition of C4d can occur before complete complement activation occurs. C3d deposition only occur after complete complement activation (Figure A). As such C3d may be a better marker of clinically significant antibody deposition.

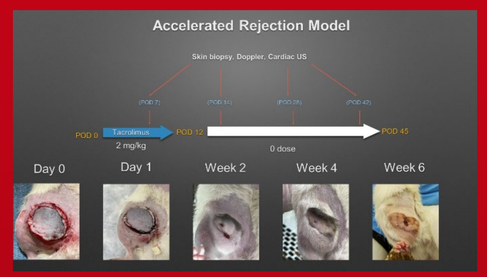
We hypothesize that another complement activation product C3d may be a more specific marker of antibody mediated rejection than C4d in recipients of skin containing VCA grafts.

Our study was conducted with the use an allogeneic Brown Norway (BN) to Lewis (Lew) rat osteomyocutaneous (OMC) hind limb vascularized flap as an experimental model of VCA.



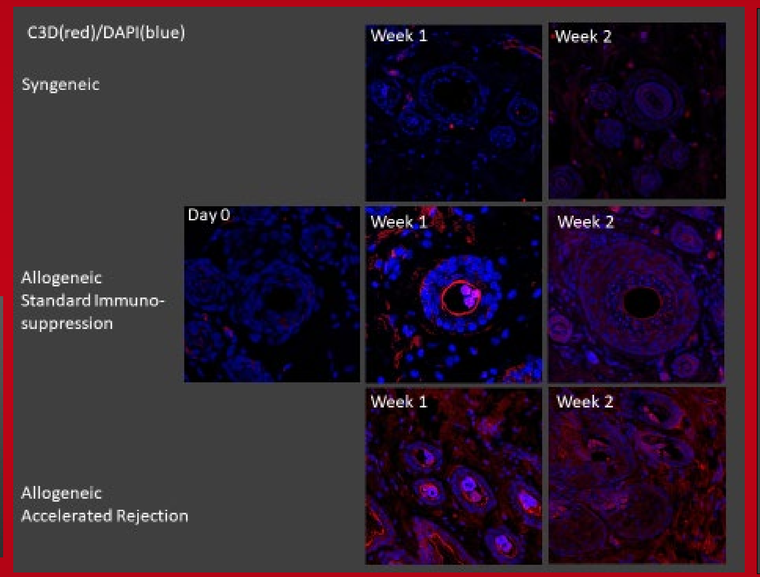
Syngeneic transplants use Lewis strain donors to Lewis recipients and do not require tacrolimus as no alloantigen rejection response is generated



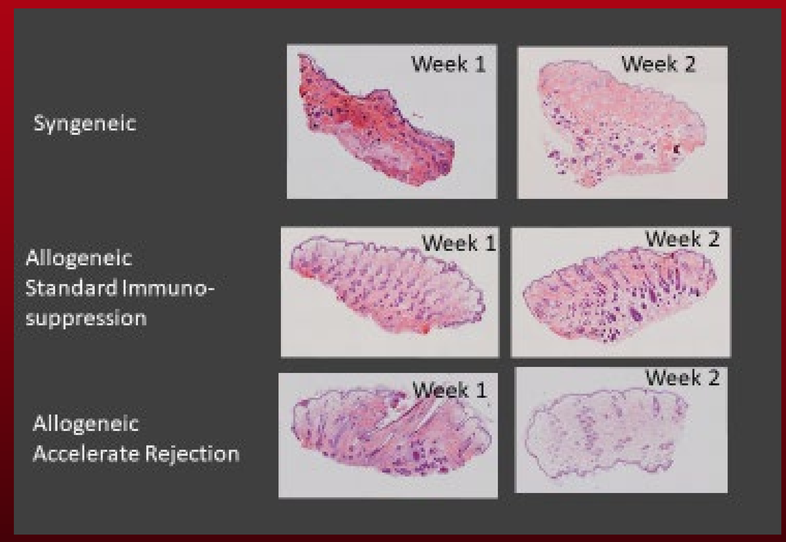


3. To prevent rejection, allogeneic recipient animals received daily shown.
4. Skin, muscle, and vascular samples were collected upon terminat

Results



H&E Staining of Skin biopsies



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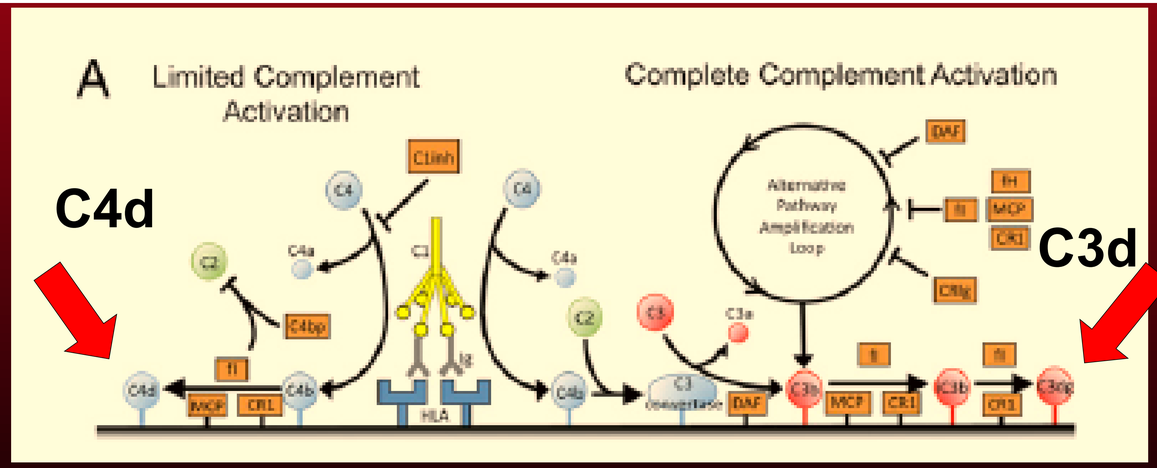
Objective:
We hypothesize that C3d may be a more specific marker of antibody mediated rejection than C4d in recipients of skin containing VCA grafts as it does not stain skin non-specifically. In addition, we hypothesize that C3d may also be a good biomarker of acute cellular rejection, unlike C4d.



Stites et al, J Immunol 2015, 195:5525

Acknowledgements

Research supporte
Program (RTRP), u