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TITLE: Regulatory T Cell-Enriching Microparticles for Promoting Vascularized Composite Allotransplant Survival

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14. ABSTRACT

The purpose of this work is to investigate the ability of engineered biomimetic drug delivery systems to prevent rejection and promote immunological tolerance in the context of composite tissue allotransplantation (CTA). For this reporting period our goals were to fabricate and characterize microparticles to be used in animal surgeries, achieve IACUC/ACURO approval for animal work and start the first phase of rodent hindlimb transplants. Particles containing IL-2,TGFβ, and rapamycin were fabricated and this triple cocktail, along with all pairwise iterations of the three components were tested for their ability to prevent hindlimb rejection (via 2 subcutaneous injections). Data at present seems to suggest that the combination of all three factors yields the best results, however some groups are still in the follow up period and accordingly, no statistical significance can be claimed until the follow up period is completed (this will happen within the next month).

15. SUBJECT TERMS
Microparticle, Regulatory T Cell, CTA, Transplant, Biomimetic

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8. Special Reporting Requirements…… N/A

9. Appendices…… N/A

Introduction

Millions of Americans have sustained unsalvageable tissue loss secondary to trauma, sepsis/disease, cancer, and congenital defects. In most cases, current reconstructive strategies are sub-optimal or fail to provide optimal results in terms of aesthetic or functional outcomes. For these patients, composite tissue allotransplantation (CTA), encompassing transplantation of hands and face is becoming an increasingly explored strategy with nearly 200 different types of clinical transplants performed over the past decade. Despite promising results and advances in microsurgical approaches, graft rejection and the deleterious effects of lifelong, high-dose, multi-drug immunosuppression have prevented the broader clinical application of CTA. This project will investigate the potential of using biomimetic microparticles to promote long-term CTA survival in the absence of systemic immunosuppression via the in situ recruitment and expansion of a patient's own suppressive regulatory T cells. These particles, referred to as Expansion MP (IL2, TGF, and Rapamycin microparticles) and Recruitment MP (CCL22 loaded microparticles) will be tested in both rodent and swine models of CTA. Finally, we will investigate immunobiological mechanisms behind any of the observed effects these particles have on allograft survival.

Key Words

CTA	Composite Tissue Allotransplantation
VCA	Vascularized Composite Allotransplantation
DC	Dendritic Cell
Treg	Regulatory T cell
FoxP3	Forkhead Box P3
TGF-β	Transforming Growth Factor Beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IFN-g	Interferon Gamma
Rapa	Rapamycin
CCL22	Chemokine Ligand 22
CCR4	Chemokine Receptor 4
MP	Microparticle
GMP	Good Manufacturing Practices
PLGA	Poly (lactic-co-glycolic) acid
ALS	Antilymphocyte Serum
ELISA	Enzyme Linked Immunosorben Assay
PCR	Polymerase Chain Reaction
IHC	Immunohistochemistry
MHC	Major Histocompatibility Complex

Accomplishments During Year 2

What were the major goals of the project during year 2?

The following goals/tasks have been completed and/or are in progress.

1. Fabrication of Recruitment and Expansion MPs that release factors as described in the project narrative (Months 1-6): This goal has been completed and was reported in our year 1 annual report
    a. Obtain design parameters from Little Lab predictive model for CCL22, IL-2, TGF-β, and Rapamycin
    b. Fabricate MP formulations using design parameters dictated by our predicted model
    c. Conduct *in vitro* release studies to verify our *in silico* predicted release of CCL22, T TGF-β and Rapamycin over a 30 day period
2. Rat IACUC and ACURO approval (Months 1-6): This goal has been completed and was reported in our year 1 annual report.
    a. IACUC protocol write up and approval
    b. ACURO approval following University of Pittsburgh IACUC approval
3. Rat CTA surgery (Months 7-24): This goal is complete.

    a. Using microsurgical techniques transplant hind-limbs from Brown Norway rats to Lewis rats
    b. In appropriate groups initiate induction therapy (ALS) on days -4 and +1 and continue maintenance therapy (FK506 0.5mg/kg) for 21 days after transplantation
    c. Inject Recruitment and Expansion MP formulations subcutaneously into the transplanted hind-limb on days 0 and +21
4. Daily monitoring of CTA (Months 6-24): This goal is complete.
    a. Monitor transplanted CTA daily for rejection using the following scale Grade 0 (no rejection), Grade I (edema), Grade II (erythema), Grade III (epidermolysis) and Grade IV (necrosis and mummification)
    b. Grafts surviving for greater than 100 days will be considered long term survivors
5. Immunohistochemistry (Months 24-30): This goal will be completed in year 3.
    a. Skin and muscle biopsies will be taken from rejecting animals and long term survivors at the time of sacrifice, formalin fixed and embedded in paraffin.
    b. To assess intragraft Treg infiltration, biopsies will be stained using immunohistochemical techniques for CD3, CD4, and FoxP3.
6. Analyze cytokine gene expression in CTA grafts via PCR (Months 24-30): This goal is ~40% complete and will be completed in year 3.
    a. Intra graft gene and cytokine expression profiles will be analyzed for all groups using real time PCR for IFN-g, TNF, IL-17, perforin, and granzyme B as well as anti-inflammatory markers such as IL-10, CTLA4, and Foxp3. A GAPDH primer and VIC labeled probe will be used as internal controls.
7. Demonstrate that our biomimetic MP therapies promote both in vitro and in vivo donor specific tolerance (Months 15-29): This goal is ~50% complete and will be fully completed in the early months of year 3.
    a. MLRs to demonstrate *in vitro* donor specific tolerance
    b. MLRs using Treg depleted tissue to demonstrate that our MP therapies promote *in vitro* donor specific tolerance that is Treg mediated.
    c. Secondary non-vascularized skin grafts performed on long-term survivors to demonstrate *in vivo* donor specific tolerance
8. Further Biological Analysis (Months 19-33): This goal is ~30% complete and will be fully completed in year 3.
    a. PCR on both transplanted hindlimb skin and naïve contralateral limb skin to demonstrate expression of anti-inflammatory and tolerogenic cytokines and transcription factors.
    b. At the time of sacrifice, blood, lymph nodes and spleen will be collected and subject to flow cytometric analysis of key intracellular cytokines and transcription factors including FoxP3, IFNγ, IL-10, and IL-17

The following goals/tasks have not begun yet and will take place in year three.

9. Fabrication of Recruitment and Expansion MP Therapies for Swine CTA Model (Months 13-33): This goal will be completed in year 3.
10. IACUC and ACURO Approval for Swine Surgeries (Months 20-26): We are currently writing our IACUC for the planned large animal study. This will be compelted in the near future.
11. Conduct swine gracilis myocutaneous free flap allotransplantation with Recruitment MP and Expansion MP Treatment (Months 26-34): This will be completed in year 3.
12. Daily monitoring of CTA grafts (Months 26-36): This will be completed in year 3
13. Biological Analysis of Tissue from Swine CTA Transplants (Months 29-34): This will be completed in year 3.

What was accomplished under these goals?

Rat Hind Limb CTA with Expansion MP Therapy Methodology

We employed a rodent hind limb transplant model to test our hypothesis that our mimetic microparticle systems can promote VCA tolerance. All animals in all groups received the same baseline immunosuppression protocol this protocol consisting of 21 days of systemic FK-506 (IP injections) at a concentration of 0.5mg/kg as well as two 0.5cc IP injections of ALS induction therapy on days -4 and +1 relative to the transplant (Figure 1). This baseline immunosuppression protocol is used to provide the necessary support for animals in the acute postoperative period and will (most importantly) demonstrate that our technologies can be used in tandem with conventional immunosuppression. Animals receiving microparticle injections received them perioperatively (day 0) and again at postoperative day 21.

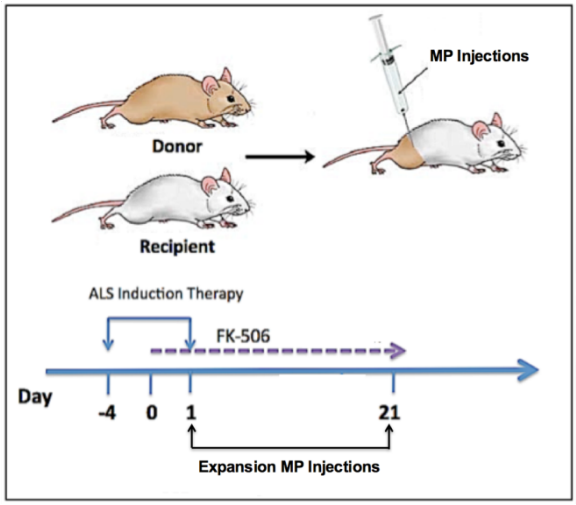

Figure 1: Therapeutic Timeline for Hind Limb Transplantation

Surgical Follow Up - Methodology

All controlled release treatments will be injected subcutaneously using a 25G insulin syringe at the time of transplantation and again on postoperative day 21. To assess rejection, animals were monitored daily and scored for rejection (appearance grading) based on physical examination. Animals were given a daily score using the following scale: Grade 0 (no rejection), Grade I (edema), Grade II (erythema and edema), Grade III (epidermolysis) and Grade IV (necrosis and "mummification"). Grafts were considered rejected when displaying signs of progressive Grade III rejection.

Surgical Follow Up – Expansion MP Key Results

While the bulk of Expansion MP therapies were tested in the previous annual report, we will include all groups in our summary for context, groups highlighted in yellow were completed in year 2, whereas all other groups were completed in year 1. For this study 0.3ml of each factor of Expansion MP (IL2MP, TGFMP, and RapaMP) was injected at a concentration of 10mg/ml. Finally, because Expansion MP is a triple cocktail that acts as a local therapy it was imperative to test all iterations of the components of Expansion MP to determine the necessary and minimal combination of factors needed to generate immunological tolerance. Thus, the following groups were tested: 1) Expansion MP, 2) IL2MP, 3) TGFMP, 4) RapaMP, 5) IL2MP+TGFMP, 6) IL2MP+RapaMP, 7) TGFMP + RapaMP, 8) Expansion MP injected in the contralateral limb, and 9) no microparticle treatment (FK506/ALS only). Limbs were monitored daily for signs of rejection and were sacrificed when limbs demonstrated visual epidermolysis or when animals reached their experimental endpoint (>150 days with no rejection). Gross observations were confirmed via hematoxylin and eosin (H&E) staining of skin biopsies (Figure 2).

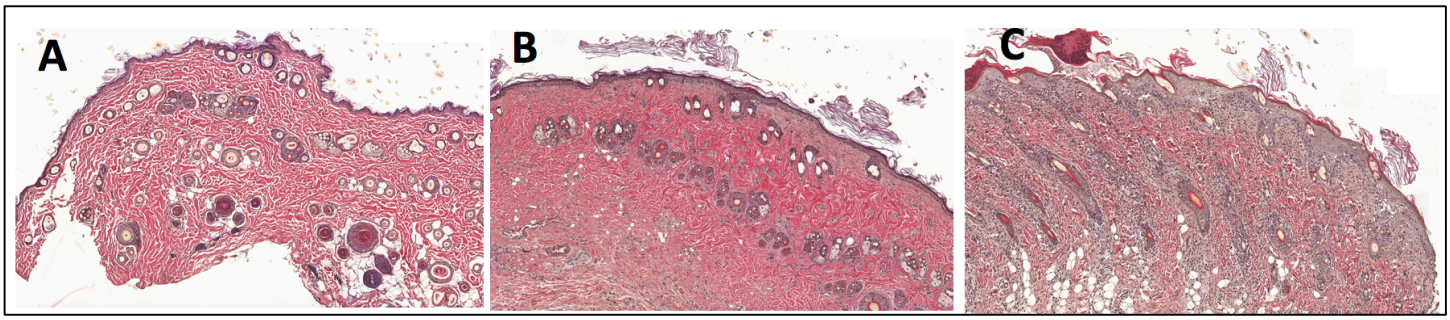

Figure 2: Representative photomicrographs of histology (H&E staining) of skin biopsies from A naïve animal, B Expansion MP treated, C actively rejecting control (10x).

The results to date are summarized below in Table 1. Because all groups receive the same baseline immunosuppression protocol for 21 days, animals under the cover of systemic FK506 should not demonstrate signs of rejection until the FK506 is discontinued at day 21. Our results strongly suggest that the triple cocktail of IL2MP, TGFMP, and RapaMP (Expansion MP), and not any other iteration of these factors is most effective at preventing VCA rejection in a rat model. Moreover, it is the local effect of Expansion MP that appeard to be responsible for this effect as evidence by the inability of Expansion MP to confer long term survival when injected in the contralateral limb (Table 1).

Table 1: Expansion MP Survival Summary

Treatment Received	Number	Onset of Rejection
Expansion MP	13	>150x12, 45
FK506/ALS	5	29, 31, 35, 38, 38
Blank MP	4	27, 28, 33,38
IL2MP	5	32, 35, 38, 40, 39
TGFMP	5	35, 38, 38, 40, 80
RapaMP	7	28, 32, 38, 38, >150 x3
TGFMP + RapaMP	6	39, 41, 43, 44, >150x2
TGFMP + IL2MP	5	39, 39, 40, 44, >150
RapaMP + IL2MP	6	38, 39, 42, 44, 44, >150
Contralateral Expansion MP	5	40, 47, 52, 67, 108

Analysis of Gene Expression in VCA Skin Biopsies

Gene expression profiles of both regulatory and inflammatory markers were evaluated in the skin of long term survivors, actively rejecting and naïve (self) rats. Total RNA was extracted from skin biopsies using TRI-reagent according to the manufacturer's instructions, and quantified using a NanoDrop 2000. For each reverse transcriptase assay, 4 µg RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit. Quantitative real-time PCR was then performed using VeriQuest Probe qPCR Mastermix, according to the manufacturer's instructions, with 5′ nuclease PrimeTime qPCR assays specific for IFNγ, FoxP3, TNF, Perforin, IL-10, Serglycin, IL-17 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, endogenous control). Duplex reactions (target gene + GUSB) were run and analyzed on a StepOnePlus Real-Time PCR System. Relative fold changes of IFNγ, FoxP3, TNG, Perfoin, IL-10, Serglycin, and IL-17 expression were calculated and normalized based on the $2^{-\Delta\Delta Ct}$method, skin biopsies from naïve animals or contralateral limbs serving as untreated controls.

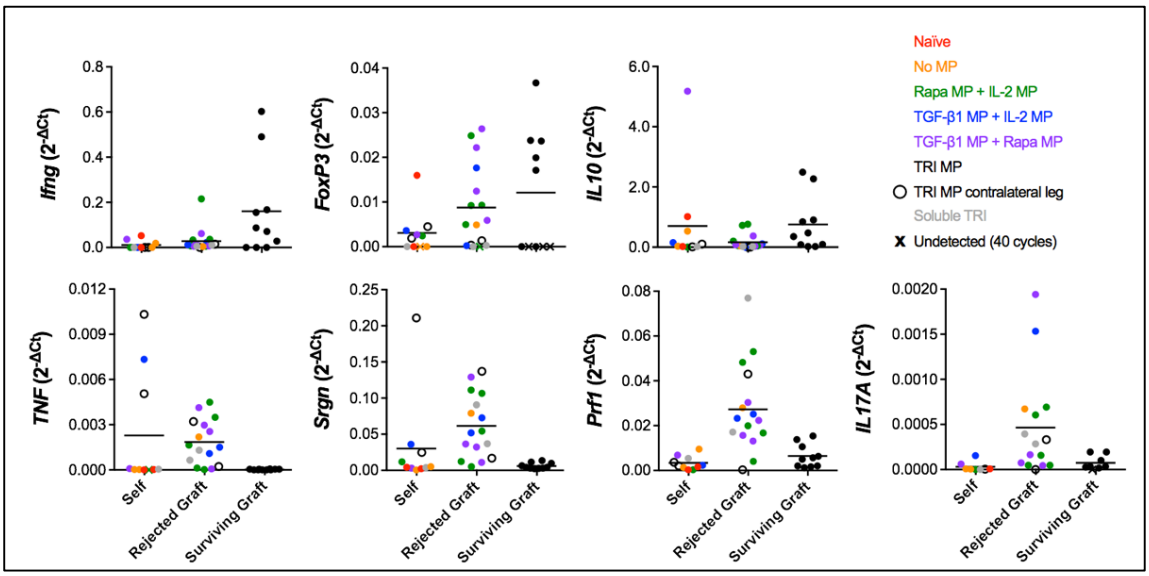

Figure 3: Gene Expression in Cutaneous Skin Biopsies

Flow Cytometric Analysis of Blood (PBMC) and Lymphoid Tissue

Mononuclear cells from blood and draining lymph nodes of long term surviving (LTS), actively rejecting and naïve animals were stained with two characteristic intra cellular markers – FoxP3 and IFNγ. Staining of FoxP3 was slightly elevated in both the blood and draining lymph nodes long term survivors when compared to naïve and rejecting animals. Interestingly, staining for IFNγ was also increased in blood and lymph nodes of long term survivors when compared to naïve and rejecting animals (Figure 4) .

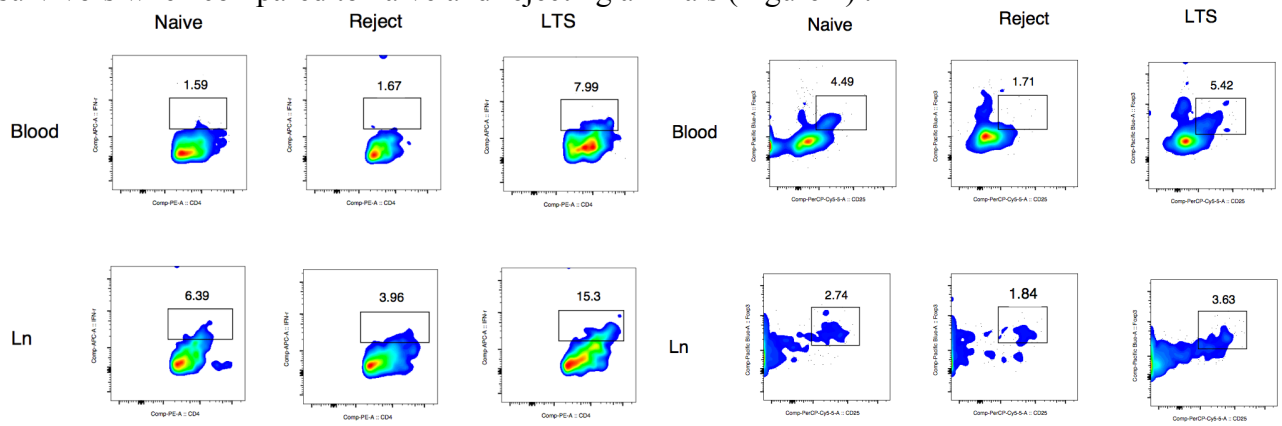

Figure 4: Intracellular INFγ (left three panels) and FoxP3 (right three panels) staining from blood (PBMC) and draining lymph nodes.

Mixed Lymphocyte Reactions: Proliferation and Suppression Assays

To test for immune hypo-responsiveness and donor specific tolerance mixed lymphocyte reactions using spleens of long term surviving (LTS), actively rejecting, and naïve rats were performed. Specifically, single-cell suspensions were prepared from spleens, and red blood cells (RBC) were lysed using RBC lysis buffer. CD4+ T cells were isolated by CD4 T cell enrichment columns according to the manufacturer's instructions (Milyteni Biotech). CD4+CD25low and CD4+CD25hi populations were sorted using a fluorescence-activated cell sorter. Proliferation capacity was assessed by co-culturing CD4+CD25low responder cells with irradiated splenocytes from Brown Norway, Wistar Furth and Lewis rats. To quantify suppressive cell function, flow-sorted CD4+CD25hi T cells from long-term surviving, actively rejecting and naive Lewis rats were cultured with CD4+ naïve responder T cells along with irradiated splenocytes from donor (BN) or the third-party (WF) stimulators. In all experiments proliferation of responders was measured via VPD450 in flow cytometry. While this data is still being analyzed our preliminary results suggested that effector CD4+CD25low cells from long term survivors proliferate less than CD4+CD25 low cells from rejecting or naïve rats when stimulated with both Brown Norway or Wistar Furth splenocytes. Furthermore, CD4+CD25hi Tregs from long term survivors may be more effective at suppressing proliferation when compared to Tregs from both actively rejecting and naïve rats.

Secondary Skin Grafting Long Term Survivors

To test for donor-specific tolerance *in vivo*, skin grafts from LEW, BN and third party (WF) rats were transplanted on lateral flanks of Lewis long-term hind limb transplant survivors (>120 days) (n=3 per group). Full thickness skin grafts 16 mm in diameter were taken from donors. Graft beds will be prepared by excising 18 mm circles on lateral thoracic wall of recipients. Grafts were then evaluated on a daily basis and monitored for signs of rejection. Rejection was when more than 90% of the graft is lost. In all three animals, skin grafts from BN donors were accepted (as evidenced by lack of necrosis and brown fur growth), whereas WF grafts rejected and became complete eschars.

Surgical Follow Up – Recruitment MP Key Results
In addition to testing therapies aimed to induce and expand Tregs at the site of a VCA, we have also developed a microparticle based system to recruit natural Tregs by setting up a chemotactic gradient. This product, referred to herein as Recruitment MP are dissolvable PLGA microparticles, ~20µm in size that release the (Treg specific) chemokine CCL22 over a period of three weeks. To test this therapy *in vivo* we employed the same model used for Expansion MP studies, with the same baseline immunosuppression protocol (Figure X). Because the effectiveness of Recruitment MP is hinged on establishing a chemotactic gradient to home Tregs to the site of the VCA, we needed to optimize the dose of Recruitment MP administer. If the gradient is not strong enough, Tregs will not be able to be recruited over long distance, however if the dosage is too high the gradient can

Table 2: Recruitment MP Survival Summary

Treatment Received	Number	Onset of Rejection
Recruitment MP 9mg	6	34, 34, 38, 40, 44, 44
Recruitment MP 50mg	8	40, 60, >150x6
Recruitment MP 100mg	6	35, 38, 44, 47 >150x2
Blank MP 50 mg	4	29, 33, 38, 38
Soluble CCL22 (50 mg equiv.)	4	39, 40, 41, 43
Contralateral Recruitment MP	4	44, 44, 44, 48

become saturated, resulting in Tregs becoming angeric to the gradient. Based on pilot data in this model and previously published data using Recruitment MP in both large and small animal models of gum disease we decided to test three different dosages of Recruitment MP: 9mg, 50mg and 100mg of microparticles. All microparticle formulations were fabricated using an initial loading concentration of 25µg CCL22/200mg PLGA. As can be seen from the raw data in Table 2, the 50mg dose of Recruitment MP was most effective at preventing hind limb rejecting with 6/8 animals going on to become long-term survivors. Finally, we also included the necessary controls in our study including blank microparticles, Recruitment MP injected in the contralateral limb (to demonstrate that our therapy works locally), and soluble CCL22 injected in the transplanted limb (Table 2).

What opportunities for training and professional development has the project provided? Nothing to Report.

How were the results disseminated to communities of interest? Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
During the next reporting period, we will continue to process tissue from long term surviving animals in both Recruitment MP and Expansion MP studies. Fresh tissue (namely blood, LN and splenocytes) will be analyzed via flow cytometry and qRT-PCR for both pro and anti-inflammatory markers. We will also continue to test for donor specificity via proliferation and suppressor assays. Finally, we are in the process of finalizing our large animal study IACUC protocol. Upon IACUC and ACURO approval, we will begin our large animal (swine) study.

Impact
What was the impact on the development of the principal discipline(s) of the project? Nothing to Report

What was the impact on other disciplines? Nothing to Report

What was the impact on technology transfer? Nothing to Report

What was the impact on society beyond science and technology? Nothing to Report

Changes/Problems

Nothing to Report.

Products

Publications, conference papers, and presentations

1. **Fisher JD**, Schweizer, R, Unadkat JV ,Fries, A, Komatsu C, Oksuz S, Solari MG, Davis M, **Gorantla VS, Little SR**. Biomimetic Microparticles can Establish Dominant Tolerance in Vascularized Composite Allotransplantation via Endogenous Regulatory T Cell Enrichment. 15th Annual McGowan Institute Retreat, Nemacolin Woodlands, PA March 6-8 2016. (Oral and Poster Presentation)

2. **Fisher JD**, Zhang W, Schweizer R, Dong L, Aral A, Zhang Z, Komatsu C, Erubas v, Unadkat J, Diaz-Perez J, Solar M, **Gorantla VS, Little SR.** Regulatory T Cell Enriching Microparticles for Promoting Vascularized Composite Allotransplant Survival. Controlled Release Society 2017 Annual Meeting, Boston Massachusetts 7/15-7/18 2017

Website(s) or other Internet site(s): Nothing to Report

Technologies or techniques: Nothing to Report

Inventions, patent applications, and/or licenses: Nothing to Report

Other Products: Nothing to Report

Participants and Other Collaborating Organizations

What individuals have worked on the project?

Name:	Steven Little
Project Role	PI
Researcher Identifier	0000-0002-7000-3931
Nearest person month worked	1.5
Contribution to Project	Dr. Little is responsible for leading this project. This includes all experimental planning as well as troubleshooting with respect to microparticle formulation and characterization

Name:	Vijay Gorantla
Project Role	Co-I
Researcher Identifier	0000-0003-0686-059X
Nearest person month worked	1.2
Contribution to Project	Dr. Gorantla is responsible for experimental design for this project and trouble shooting especially with respect to animal CTA and immunobiology.

Name:	James Fisher
Project Role	Graduate Student
Researcher Identifier	
Nearest person month worked	12
Contribution to Project	Mr. Fisher is responsible for fabrications of all microparticles used in this project as well as surgical assistance and long term animal follow up.
Funding Support	DoD Award W81XWH-15-1-0244

Name:	Ali Mubin Aral
Project Role	Postdoctoral Fellow
Researcher Identifier	
Nearest person month worked	4
Contribution to Project	Dr.Aral is an experienced microsurgeon who completed a portion of the hindlimb transplants during this reporting period.
Funding Support	Internal/Departmental Funds

Name:	Zhaoxiang Zhang
Project Role	Postdoctoral Fellow

Researcher Identifier	
Nearest person month worked	2
Contribution to Project	Dr. Zhang is an experienced microsurgeon who completed a portion of the hindlimb transplants during this reporting period
Funding Support	Internal/Departmental Funds

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to Report.

What other organizations were involved as partners? Nothing to Report.