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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). A microparticle-based system engineered to release the Treg-recruiting chemokine CCL22 and particles containing IL-2, TGFb , and rapamycin were fabricated and this triple cocktail was tested for their ability to prevent hindlimb rejection. Recruitment-MP prolonged hindlimb allograft survival indefinitely (>200 days) and promoted donor-specific tolerance. Recruitment-MP treatment enriched Treg populations in allograft skin and draining lymph nodes, and enhanced Treg function without affecting the proliferative capacity of conventional T cells. TRI-MP prolongs rat hindlimb allograft survival indefinitely without long-term systemic immunosuppression. We further demonstrate that this form of local immune therapy imparts systemic, antigen-specific tolerance to hindlimb allograft recipients. Ultimately, this approach has the potential to significantly impact the field of VCA and reconstructive transplantation by minimizing the need for the sustained, multi-drug systemic immunosuppression with its associated long-term toxicity.

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

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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 (VCA), 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 VCA. This project will investigate the potential of using biomimetic microparticles to promote long-term VCA 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 TRI-MP (**Tr**eg **I**nducing **M**ircoparticles, containing **I**L2, **T**GF, and Rapamycin microparticles) and Recruitment MP (CCL22 loaded microparticles) will be tested in both rodent and swine models of VCA. 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 4

What were the major goals of the project during year 4?

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 completed and was reported in our year 3 annual report.
    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 completed and was reported in our year 3 annual report.
    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 is complete.
    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 complete.
    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 complete.
    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 complete.
    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
9. Fabrication of Recruitment and Expansion MP Therapies for Swine CTA Model (Months 13-33): This goal has been completed. We will continue to manufacture microparticles as needed throughout our large animal study
10. IACUC and ACURO Approval for Swine Surgeries (Months 20-26): IACUC protocol has been approved at ACURO application has been submitted
11. Write up and dissemination of results obtained in Aims 1 and 2. This goal is complete.

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

1. Fabrication of Recruitment and Expansion MP Therapies for Swine VCA
2. IACUC and ACURO Approval for Swine Surgeries at WFIRM
3. Conduct Swine Gracilis Myocutaneous Free Flap Allotransplantation with Recruitment MP and Expansion MP Treatment. This will be completed in the NCE.

4. Daily monitoring of VCA grafts. This will be completed in the NCE
5. Biological Analysis of Tissue from Swine VCA Transplants. This will be completed in the NCE
6. Data analysis, interpretation of results, comprehensive statistical analysis of study outcomes. This will be completed in the NCE
7. Prepare final study report and publication of results. This will be completed in the NCE

What was accomplished under these goals?

This report will describe two approaches to prolong rodent (hindlimb) VCA survival via local enrichment of Regulatory T cells (Treg). In the first portion of this work it is demonstrated that microparticles that release the Treg recruiting chemokine CCL22 (Recruitment-MP) are able to prolong graft survival indefinitely (>200 days) in a rodent model of hind-limb transplantation. Recruitment-MP also leads to a significant decrease in the expression of proinflamatory cytokines in the skin and draining lymph nodes of graft recipients.

More so, data suggest that Recruitment-MP are even capable of imparting antigen-specific tolerance to allograft recipients, as evidenced by *ex vivo* proliferation assays and secondary skin grafting. Taken as a whole, this study demonstrates the unique ability of Recruitment-MP to reduce inflammation and restore homeostasis in an aggressive model of allotransplantation.

In the second portion of this work it is demonstrated that TRI-MP is able to prolong hind limb survival (>300 days in 11/12 anaimals) in the abscense of long term systemic immunosuppression.

Furthermore, intragraft skin biopsies as well as draining lymph nodes from TRI-MP treated long term surviving grafts exhibit significantly lower expression of proinflamatory cytokines (TNF, IL-17, Serglycin, Perforin-1 and IFN) compared to skin and draining lymph node samples taken from actively rejecting controls.

Moreover, TRI-MP increases the percentage of FoxP3+ Tregs and decreases the percentage of $IFN\gamma^{+}CD4^{+}$ cells in the draining lymph nodes of TRI-MP treated animals.

More so, data suggest that CD4+CD25+ Tregs isolated from TRI-MP treated animals exhibit superior suppressive function and donor antigen specificity.

Besides, data also show that TRI-MP is able to promote donor antigen specific tolerance in vivo. Specifically, TRI-MP recipients with long term surviving grafts (>200 days, n=3) were subject to secondary, non-vascularized skin grafting from both Brown Norway (donor) and Wistar Furth (third party) recipients. The long term survivors accept Brown Norway grafts, while failing to accept Wistar Furth skin grafts.

Finally, we are happy to report that the findings obtained in both Aims 1 and 2 of this proposal have been compiled into two separate manuscripts. Our findings with Recrutiment-MP is currently under revision at *Science Advances* and our work with Expansion-MP (now referred to as TRI-MP) has been accepted for publication in the *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*.

Methodology

Hind-Limb Transplantation

Using techniques developed in the University of Pittsburgh's Department of Plastic Surgery, hind limbs from donor Brown Norway Rats were transplanted to donor Lewis Rats. Specifically, donor femoral vessels were

anastomosed end-to-end to recipient femoral vessels. Femoral osteosynthesis was performed with an 18-gauge intramedullary rod.

Recruitment-MP Fabrication and Characterization

Poly (lactic-co-glycolic) acid (PLGA) microparticles (MP) containing recombinant mouse CCL22 (R&D systems, Minneapolis, MN) were prepared using a standard water-oil-water double emulsion procedure that has previously been des. Briefly, PLGA (RG502H, Boehringer Ingelheim, Petersburgh, VA) microparticles were prepared by mixing 200 µL of an aqueous solution containing 25 µg of rmCCL22 and 2 mg of BSA and 15 mmol NaCl with 200 mg of polymer dissolved in 4 mL of dichloromethane. The first water-in-oil emulsion was prepared by sonicating this solution for 10 seconds. The second oil-in-water emulsion was prepared by homogenizing (Silverson L4RT-A) this solution with 60 mL an aqueous solution of 2% polyvinyl alcohol (M.W. ~25,000, 98 mol. % Hydrolyzed, PolySciences, Warrington, PA) for 60 seconds at 3000 RPM. This solution was then mixed with 1% polyvinyl alcohol and placed on a stir plate agitator for 3 hours to allow the dichloromethane to evaporate. The microspheres were then collected and washed 4 times in deionized (DI) water, to remove residual polyvinyl alcohol, before being re-suspended in 5 mL of DI water, frozen, and lyophilized for 72 hours (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 100mTorr).

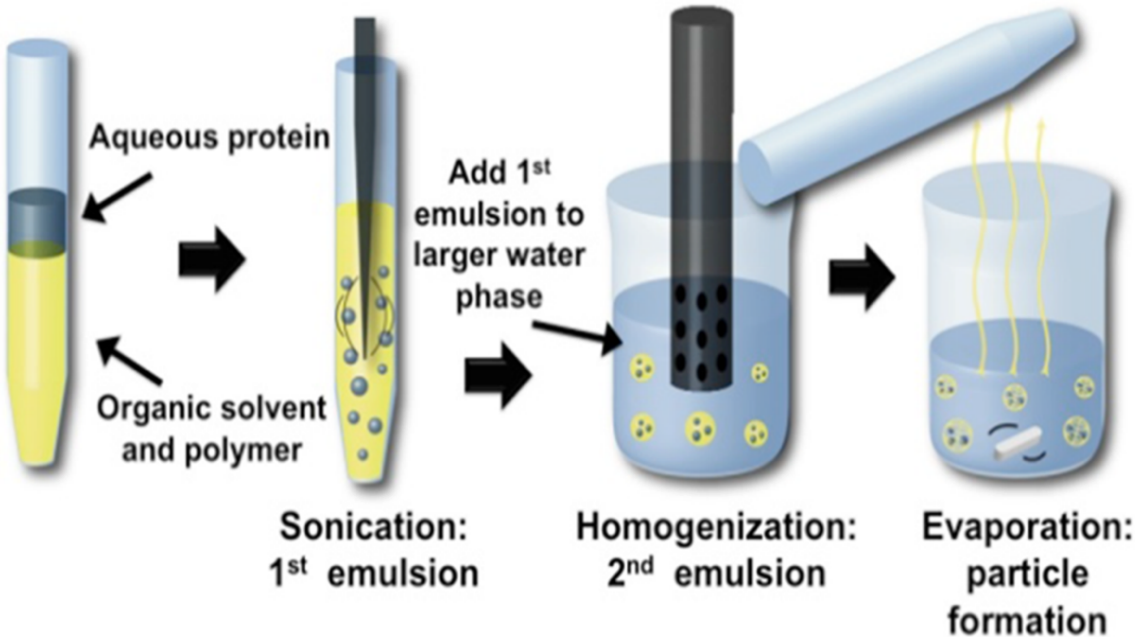

Figure 1. Recruitment-MP were fabricated using a water in oil in water (W/O/W) double emulsion technique.

Surface characterization of microspheres was conducted using scanning electron microscopy (JEOL JSM-6510LV/LGS) and microsphere size distribution was determined by volume impedance measurements on a Beckman Coulter Counter (Multisizer-3, Beckman Coulter, Fullerton, CA). CCL22 release from microspheres was determined by suspending 7-10 mg of microspheres in 1 mL of phosphate buffered saline (PBS) placed on an end-to-end rotator at 37°C. CCL22 release sampling was conducted at various time intervals by centrifuging microspheres and removing the supernatant for CCL22 quantification using ELISA (R&D Systems, Minneapolis, MN), sampling of releasates is shown in Figure 2 below. Microspheres were re- suspended with 1 mL of fresh PBS and returned to the rotator at 37°C.

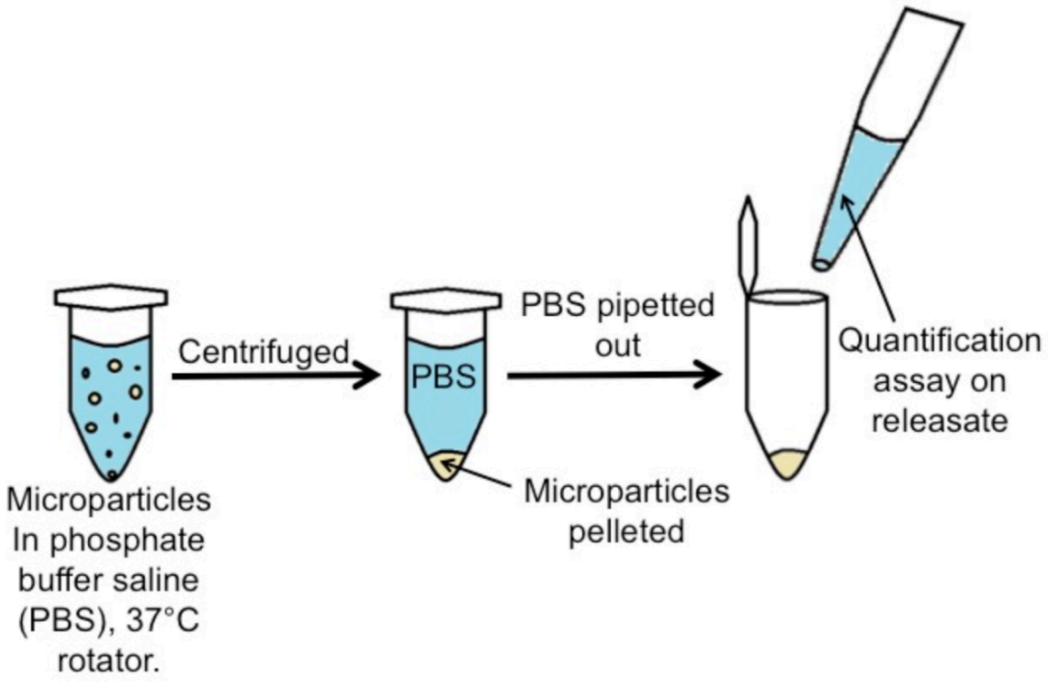

Figure 2. In vitro release of CCL22 from Recruitment-MP was measured for 40 days.

Study Design and Groups

All hind-limb recipients in all groups received the same baseline immunosuppression protocol consisting of 21 days of FK506 (LC Laboratories, Woburn, MA) at a dose of 0.5 mg/kg, injected daily intraperitoneally (I.P.). Rats also received two doses of rabbit anti-rat lymphocyte serum (Accurate Chemical, Westbury NY) injected I.P. on POD -4 and 1 (Figure 3). Microparticles were injected subcutaneously in the lateral aspect transplanted limb (unless otherwise noted). Animals receiving transplants were allocated into groups consisting of the following treatments, note all animals in all groups received the same baseline immunosuppression protocol: 1.) FK506/ALS baseline immunosuppression only (n=4), 2.) 9mg Recruitment-MP (10mg/ml, n=6), 3.) 50mg Recruitment MP (50mg/ml, n=8), 4.) 100mg Recruitment-MP (50mg/ml, n=6), 5). 50mg Blank MP (n=3), 6.) Soluble CCL22 (n=4), 7.) 50mg Recruitment-MP injected in contralateral (non-transplanted) limb (n=4).

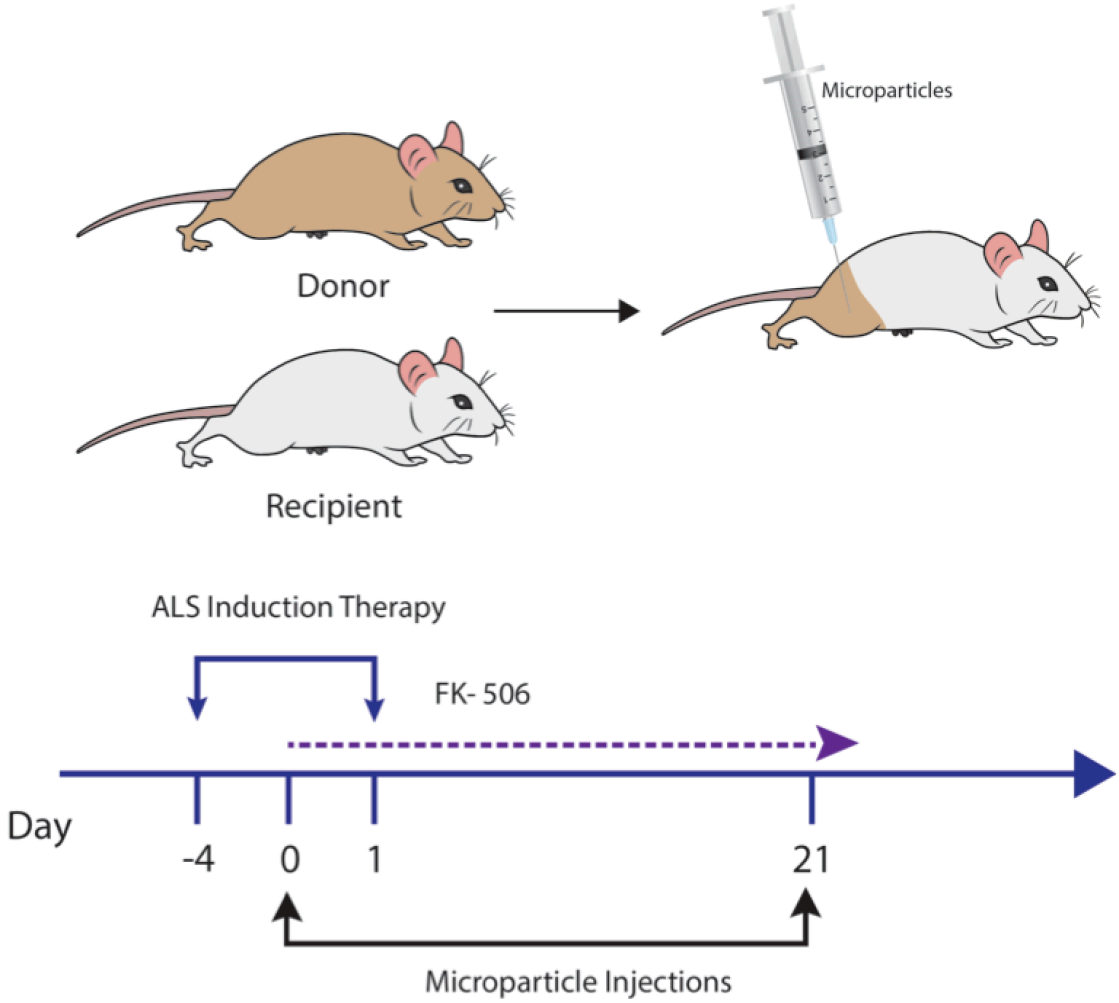

Figure 3. The experimental timeline for all animals receiving hind limb VCA. All animals receive the same baseline immunosuppression protocol consisting of 21 days of 0.5 mg/kg FK506 IP as well as two 500cc IP doeses of rabbit anti-rat lymphocyte serum. Animals receving microparticles were administered microparticles subcutaneously in the transplanted graft (unless otherwise noted) on postoperative days (POD) 0 and 21.

Hind-Limb Allograft Monitoring

To assess rejection, hind limbs were monitored daily and scored for rejection (appearance grading) based on physical examination. Limbs 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 (Figure 4).

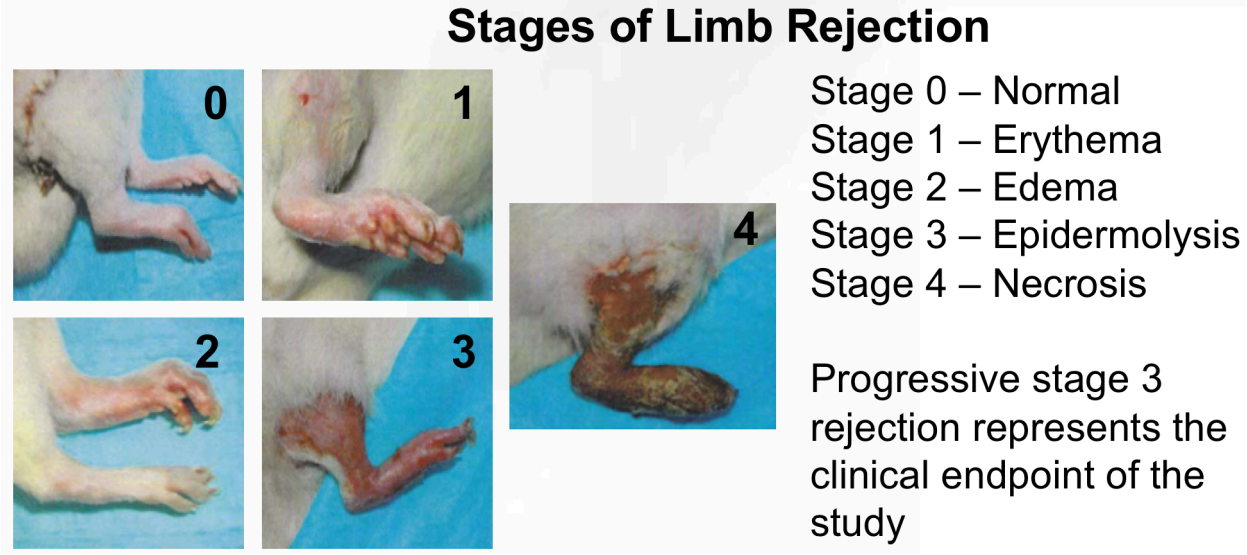

Figure 4: The clinical stages of limb rejection. Animals receiving a hind limb VCA were monitored daily and scored on a five-point rejection scale. Limbs displaying progressive stage 3 rejection were considered "rejected."

Histology

Skin and muscle samples were obtained from the transplanted limbs of animals at their experimental endpoint (progressive grade III rejection or long term survival >200days). Samples were fixed in 10% neutral buffed formalin, paraffin embedded, sectioned into 5μm slices and stained hematoxylin and eosin (H&E) for microscopic examination of tissue architecture and mononuclear infiltration.

Flow Cytometric Analysis

Draining and non-draining lymph nodes (DLN and NDLN) were harvested at their experimental endpoint (progressive grade III rejection or long term survival >200days). Lymph node samples were then processed to form a single cell suspension. Cells were stained with the following fluorescently labeled antibodies: anti-CD4 PE , anti-CD25 PerCP/Cy5.5, anti-FoxP3 Pacific Blue and anti-IFNγ APC (eBioscience, San Diego, CA). For intracellular cytokine staining, the cells were placed in a 96-well plate overnight in cell culture media with Cell Stimulation Cocktail (plus protein transport inhibitor, eBioscience) and stained with anti-IFN-γ. Stained cells were then analyzed using FlowJO (Ashland, OR).

Gene Expression and PCR

Gene expression profiles of inflammatory markers were evaluated in the skin and lymph nodes of long term survivors, actively rejecting and naïve (self) rats. Total RNA was extracted from samples 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 (Rn00594078_m1 Dye: VIC-MGB_PL), TNF (Rn99999017_m1 Dye: VIC-MGB_PL), Perforin-1 (Rn00569095_m1 Dye: VIC-MGB_PL), Serglycin (Rn00571605_m1 Dye: VIC-MGB_PL), IL-17 (Rn01757168_m1 Dye: VIC-MGB_PL) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, endogenous control, Rn99999916_s1). Duplex reactions (target gene + GAPDH) were run and analyzed on a StepOnePlus Real-Time PCR System. Relative fold changes of IFNγ, TNF, Perforin-1, Serglycin, and IL-17 expression were calculated and normalized based on the $2^{-\Delta\Delta Ct}$ method and then further normalized to naïve tissue, skin biopsies from naïve animals or contralateral limbs serving as untreated controls.

Cell Proliferation and Suppression Assays

Spleens from rats with long-term surviving hind limbs and naïve rats were processed into a single cell suspension. Red blood cells (RBC) were lysed using RBC lysis buffer (Thermo Fisher Scientific, Pittsburgh PA). $CD4^+$ T cells were isolated by CD4 T cell enrichment columns according to the manufacturer's instructions (Milyteni Biotech, Auburn CA). $CD4^+$ enriched cells were then stained with anti-CD4 PE and anti-CD25 perCP/Cy5.5. $CD4^+CD25^{low}$ (Teff) and $CD4^+CD25^{hi}$ (Treg) populations were sorted using a fluorescence-activated cell sorter. To assess proliferative function, $CD4^+CD25^{low}$ (Teff) from long-term surviving and naïve rats were stained with VPD45 (BD Biosciences, San Jose, CA) and each was co-cultured/stimulated with irradiated splenocytes harvested from Brown Norway (BN) rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using FlowJO (Ashland, OR). The proliferative capacity of Teff from long term surviving rats was normalized to that of naïve rats.

To quantify suppressive cell function $CD4^+CD25^{hi}$ (Treg) isolated from long term surviving and naïve rats were tested for their ability to suppress Teff proliferation in an MLR. $CD4^+CD25^{low}$ Teff from naïve rats were stained with VPD45 and co-cultured/stimulated with irradiated splenocytes harvested from Brown Norway (BN) rats and $CD4^+CD25^{hi}$ Tregs harvested from either long term surviving or naïve rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using FlowJO (Ashland, OR). Percent suppression was calculated using the formula:

$$Percent\ Suppression = 1 - \left(\frac{Percent\ Proliferation\ Naive\ Teff + BN\ Splenoctye + Treg}{Percent\ Proliferation\ of\ Naive\ Teff + BN\ Splenocyte}\right)$$

A final MLR was set up to test for antigen specificity in $CD4^+CD25^{hi}$ (Treg) isolated from long term surviving rats. $CD4^+CD25^{low}$ Teff from naïve rats were stained with VPD450 and stimulated with either Brown Norway (BN) or Wistar Furth (WF) irradiated splenocytes and then co-cultured with $CD4^+CD25^{hi}$ Tregs isolated from long term surviving rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using FlowJO (Ashland, OR). Percent suppression was calculated using the formula:

$$Percent\ Suppression = 1 - \left(\frac{Percent\ Proliferation\ Naive\ Teff + BN/WF\ Splenoctye + Treg}{Percent\ Proliferation\ of\ Naive\ Teff + BN/WF\ Splenocyte}\right)$$

Full Thickness Secondary Skin Grafting

Donor antigen specific tolerance was assessed *in vivo* in three long-term surviving animals from the 50mg Recruitment MP group via secondary skin graft challenge. Skin allografts were harvested from donor strain (BN) or third-party strain rats (WF) and transplanted to the long-term survivors >200 days after VCA (Figure 5). Grafts were bolstered in place for 5 days and subsequently evaluated daily for signs of rejection. Rejection was defined as necrosis of the skin graft.

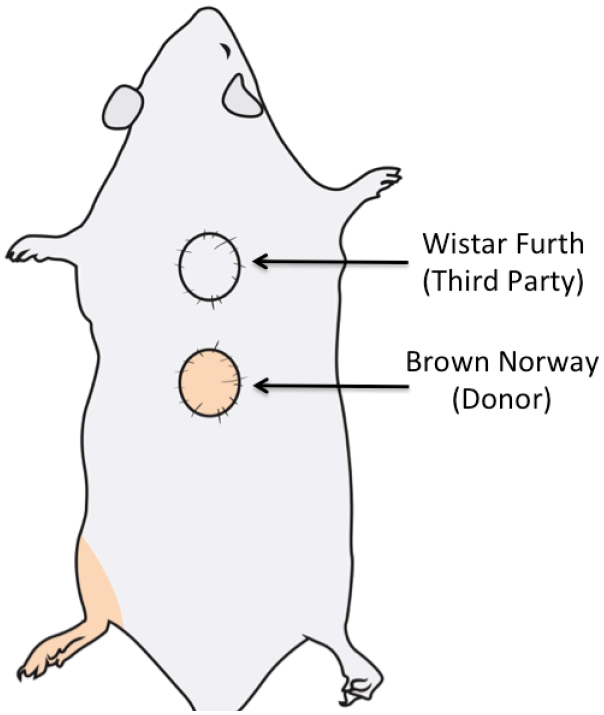

Figure 5: In vivo donor antigen specific toleranc was tested using a secondary sking graft challenge to animals with long term surviving hind limb VCAs.

Statistics

All data was expressed as mean ± standard deviation, followed by Student's t-test for two independent samples or analysis of one-way analysis of variance. The influence of various treatments on VCA survival was analyzed using a log-rank test. $p < 0.05$ was considered significant.

Results – Recruitment-MP

Characterization of Recruitment-MP

Recruitment-MP were fabricated to produce ideal CCL22 release kinetics, such that a physiological gradient of CCL22 could be established for effective Treg recruitment *in vivo*. Figure 6C demonstrates that Recruitment-MP is able to release CCL22 in a linear manner over a period of 40 days. Scanning electron micrographs of intact MP indicate that they are spherical and slightly porous (Figure 6A). The surface of Recruitment MP was specifically formulated to be porous, to allow continuous release (without periods of lag) of chemokine (Figure 6A), as guided by new mechanistic descriptions of how controlled release of proteins occurs in such systems. Further, the particles were designed to be large enough to avoid uptake by phagocytic cells and to prohibit their movement across vascular endothelium, with consequent immobilization at the site of placement (Figure 6B).

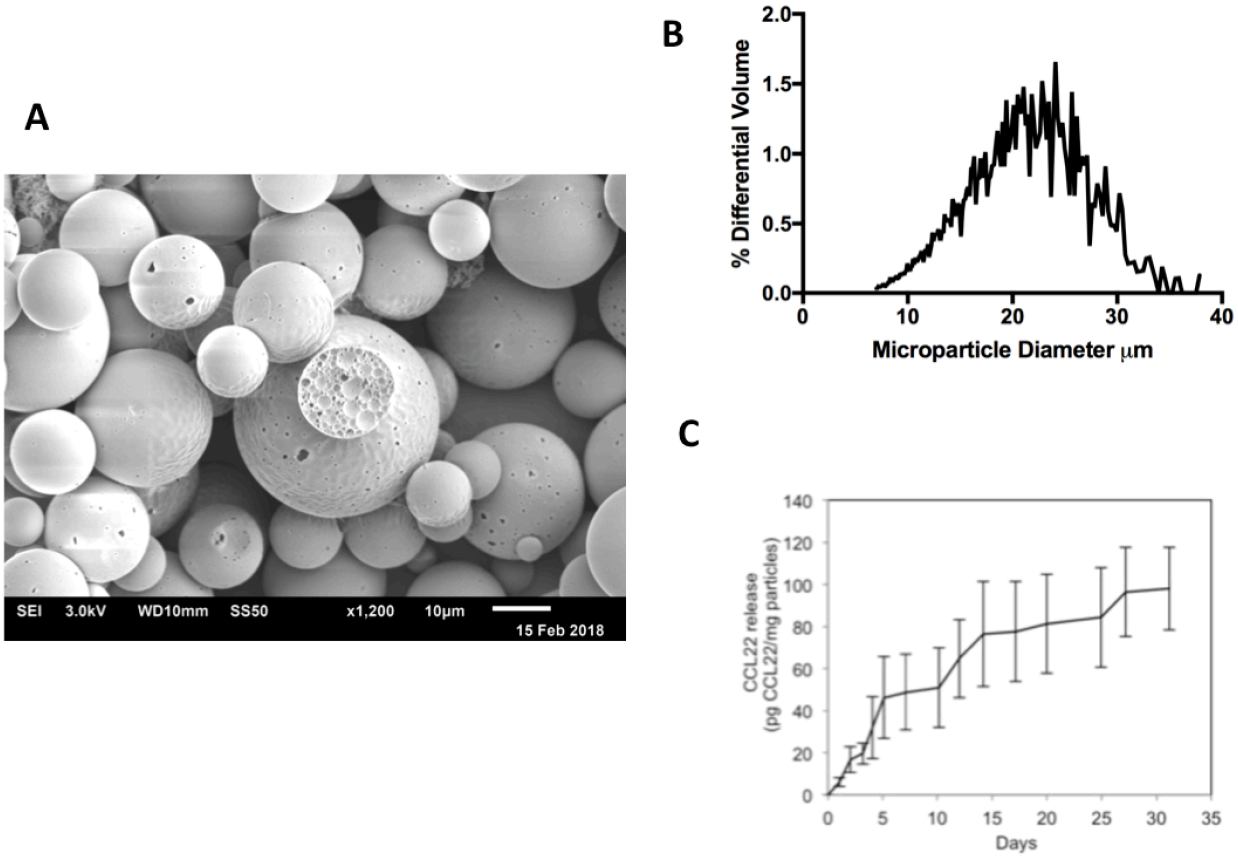

Figure 1. Characterization of Recruitment-MP. Recruitment-MP is spherical in shape and slightly porous (A) with an average particle diameter of 18.3µm (B). Release kinetics demonstrate that Recruitment-MP release CCL22 in a linear fashion for 30 days (C).

Recruitment-MP prevents rejection and promote long-term survival of hind-limb allografts.
To test the ability of Recruitment-MP to prevent graft rejection in a clinically relevant model of VCA, vascularized hindlimbs were transplanted from Brown Norway rat donors to Lewis rat recipients (complete MHC-mismatch). All animals in all groups received the same baseline immunosuppression protocol consisting of 21 days of FK-506 (Tacrolimus, 0.5 mg/kg IP daily) as well as two doses of rabbit anti-rat lymphocyte serum (ALS, 0.5cc IP on POD -4 and +1). Furthermore, all animals that received any form of microparticle treatment received two subcutaneous injections of microparticles in the transplanted grafts on POD 0 and 21 respectively. Transplanted limbs were evaluated macroscopically using the following scale: Grade 0 (no rejection), Grade I (edema), Grade II (erythema and edema), Grade III (epidermolysis) and Grade IV (necrosis). Grafts displaying signs of progressive Grade III rejection were considered "rejected."

Animals only receiving the baseline immunosuppression protocol of FK506/ALS served as our baseline for comparison. As can be seen in Figure 7A these animals reliably reject their grafts 2-3 weeks after systemic FK506 is discontinued at day 21. Three doses of Recruitment-MP (9mg, 50mg and 100mg) were tested to determine the optimal gradient necessary for recruiting Tregs and prolonging graft survival. As illustrated in Figure 7A, animals receiving 9mg and 100mg of Recruitment-MP had a graft mean survival time (MST) of 41.5 and 44.0 days respectively. However, treatment with 50mg of Recruitment-MP significantly prolonged graft survival (long term survival >200days in 6/8 animals) when compared to our baseline immunosuppression group as well as the 9mg and 100mg doses. Furthermore, animals treated with 50mg of blank Recruitment-MP (MST 39 days), soluble CCL22 (MST 37.5 days), and 50mg Recruitment-MP injected in the contralateral (non-

transplanted) limb (MST 36.0 days) did not experience any significant prolonged graft survival when compared animals only receiving the baseline immunosuppression protocol.

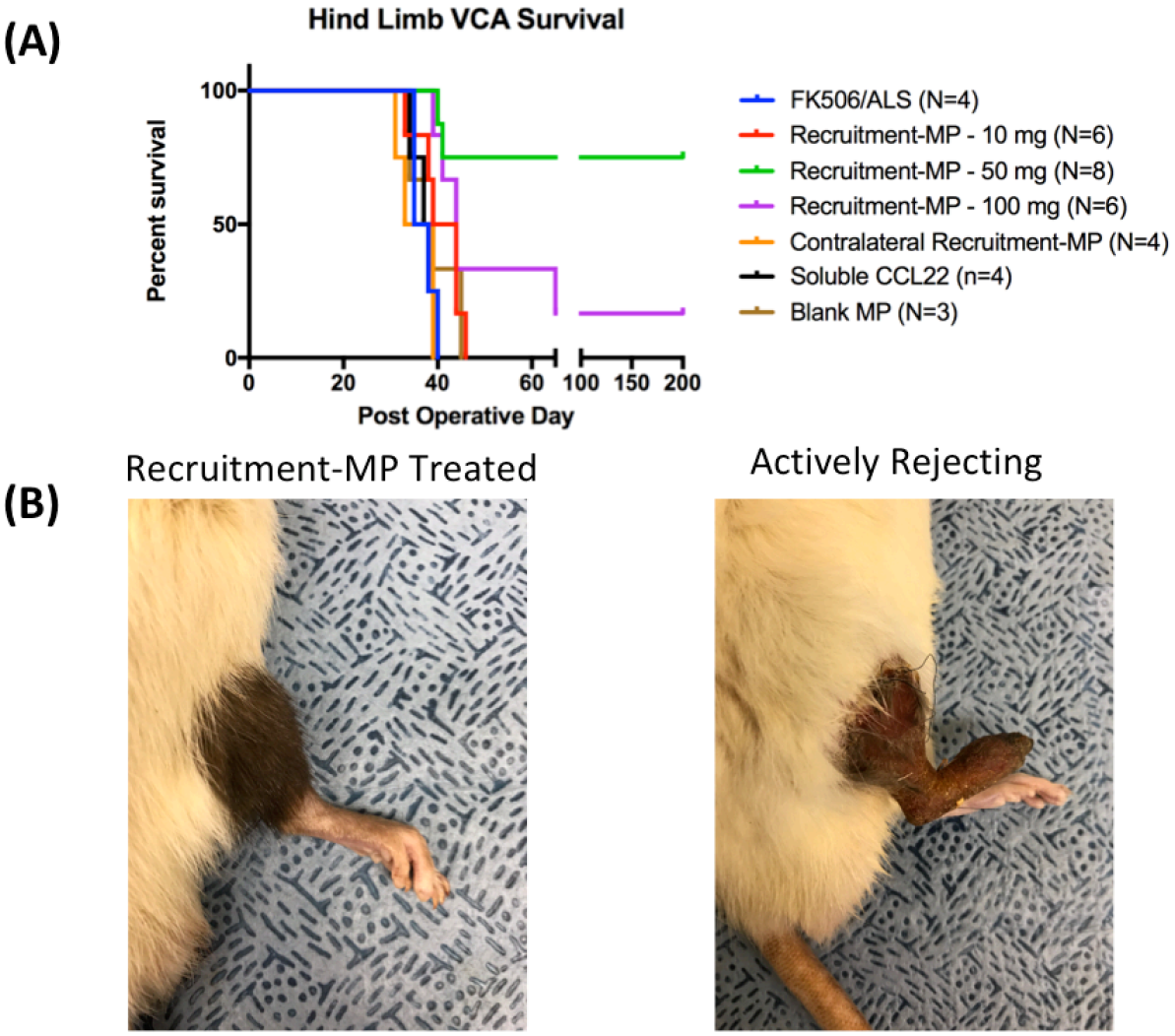

Figure 7: Treatment with 50mg of Recruitment-MP is able prolong allograft survival indefinetly in 6/8 animals. This result is statistically significant p<0.05 when compared to all controls (A).

Histologically, grafts undergoing Grade III-IV rejection showed complete obliteration of the epidermis, substantial mononuclear infiltration in the dermis and in perivascular regions (Figure 8, below). Additionally, rejecting grafts also experienced substantial myositis as evidenced by massive mononuclear infiltration in muscle tissue and disruption of muscle tissue architecture. Conversely, skin and muscle biopsies taken from animals receiving 50mg of Recruitment-MP show minimal mononuclear infiltration, and intact tissue architecture, comparable to muscle and skin biopsies taken from naïve animals (Figure 8 below).

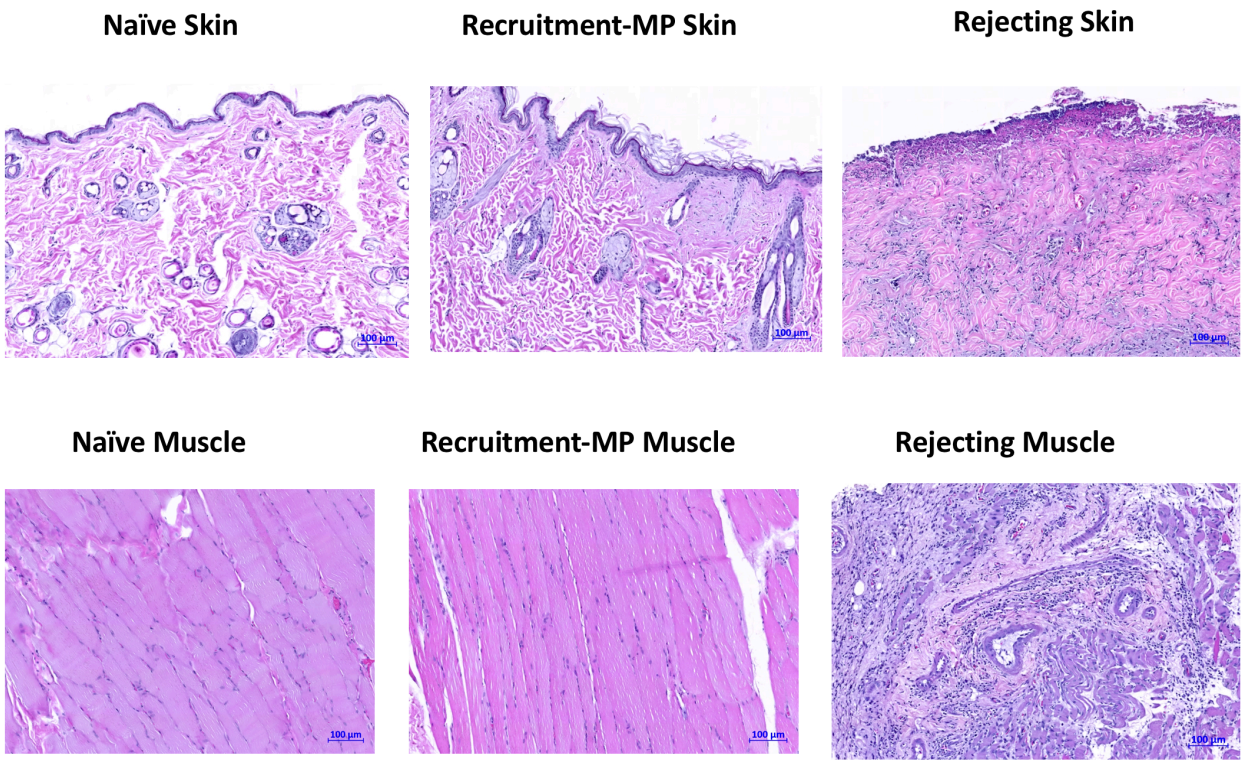

Figure 8: Recruitment-MP is able to preserve the architectural integrity of intra-graft tissues (muscle and skin). Tissue samples taken from rejecing animals display complete destruction of tissue archtecture with dense mononuclear infiltration (right pannel).

Recruitment-MP reduces the expression of pro-inflammatory mRNA in draining lymph nodes and skin biopsies of hind-limb transplant recipients.

To determine whether treatment with Recruitment-MP could suppress inflammation locally in the context of VCA, intra-graft skin samples and draining lymph nodes were harvested from actively rejecting animals (tissue harvested at Grade III-IV rejection), and TRI-MP treated VCA recipients (tissue harvested at experimental endpoint, POD >200). We then measured expression of pro-inflammatory genes TNF-α, IFN-γ, Perforin-1 and Serglycin. Expression of all 4 genes (normalized to expression in naïve tissue) was significantly decreased in skin biopsies from Recruitment-MP treated VCA recipients when compared to analogous tissue samples from actively rejecting grafts (Figure 9, below). In draining lymph nodes of Recruitment-MP treated VCA recipients expression of IFN-γ, Perforin-1 and Serglycin were significantly decreased compared to draining lymph nodes from actively rejecting animals.

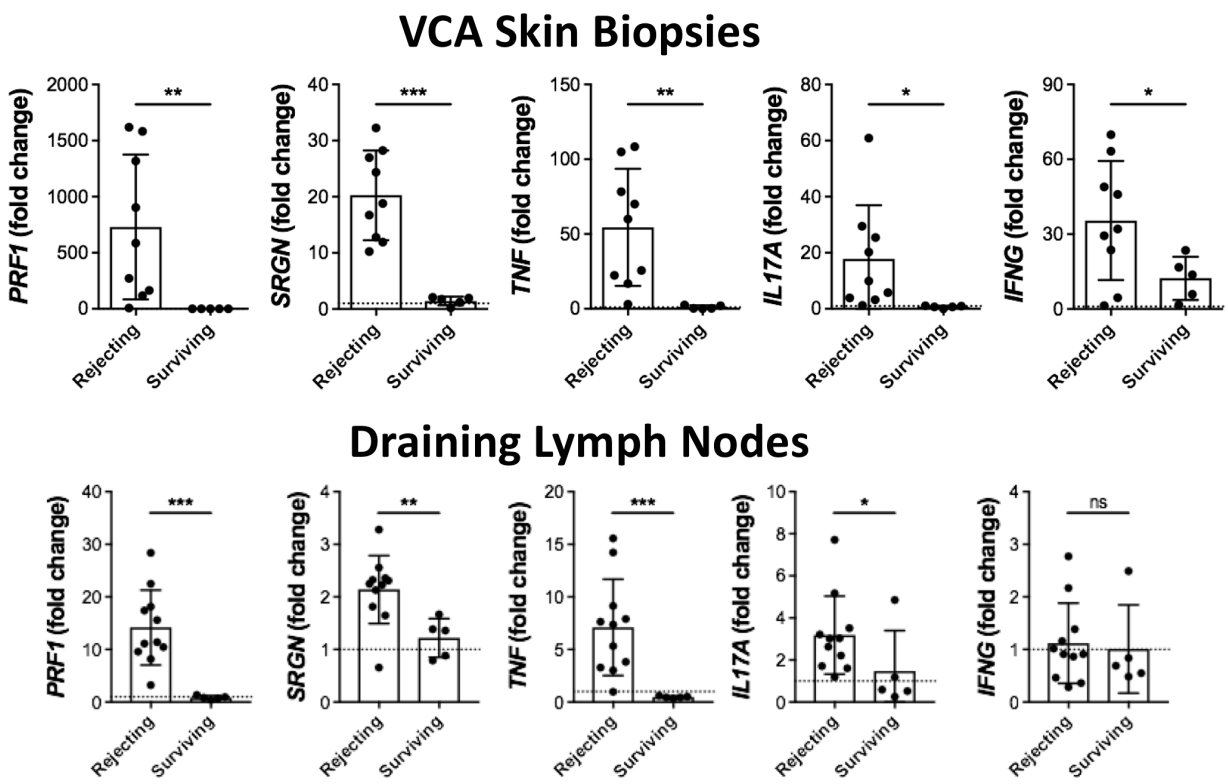

*Figure 9: Relative mRNA expression in skin samples and draining lymph nodes from rejecting hind limb allografts (n=9 for skin, n=11 for draining lymph nodes) vs. surviving Recruitment-MP treated hind limb allografts (n=5 for both skin and lymph nodes). Expression levels are presented as fold changes ($2^{-\Delta\Delta Ct}$) relative to naïve skin (N≥6). Bars represent mean ± SD, and dots represent values from individual rats. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.*

Recruitment-MP increases the number $CD4^+$ $CD25^{hi}$ $FoxP3^+$ cells in the draining lymph nodes of long term surviving allografts and tips the local immune balance toward immunoregulation.

To assess potential mechanisms behind the enhanced allograft survival associated with Recruitment-MP, local phenotypic changes in the draining lymph nodes of animals with long term surviving grafts were examined. At the experimental endpoint (POD >200days for long term survivors or Grade III rejection for rejecting grafts), draining and non-draining inguinal lymph nodes were harvested and the local helper CD4+ T cell phenotype was analyzed. Allograft draining lymph nodes from Recruitment-MP treated animals showed an increased percentage of CD4+ CD25hi FoxP3+ cells (normalized to percentages from naïve animals), when compared to both non-draining lymph nodes form Recruitment-MP treated animals as well as draining lymph nodes from actively rejecting animals (Figure 10A, below). However, allograft draining and non-draining lymph nodes from Recruitment-MP treated animals showed a significantly decreased percentage of inflammatory CD4+ IFNγ+ cells (normalized to percentages from naïve animals) when compared to draining lymph nodes from actively rejecting animals. Further, draining lymph nodes from actively rejecting animals demonstrated a significantly higher percentage of CD4+ IFNγ+ cells than from non-draining lymph nodes of actively rejecting animals (Figure 10B, below).

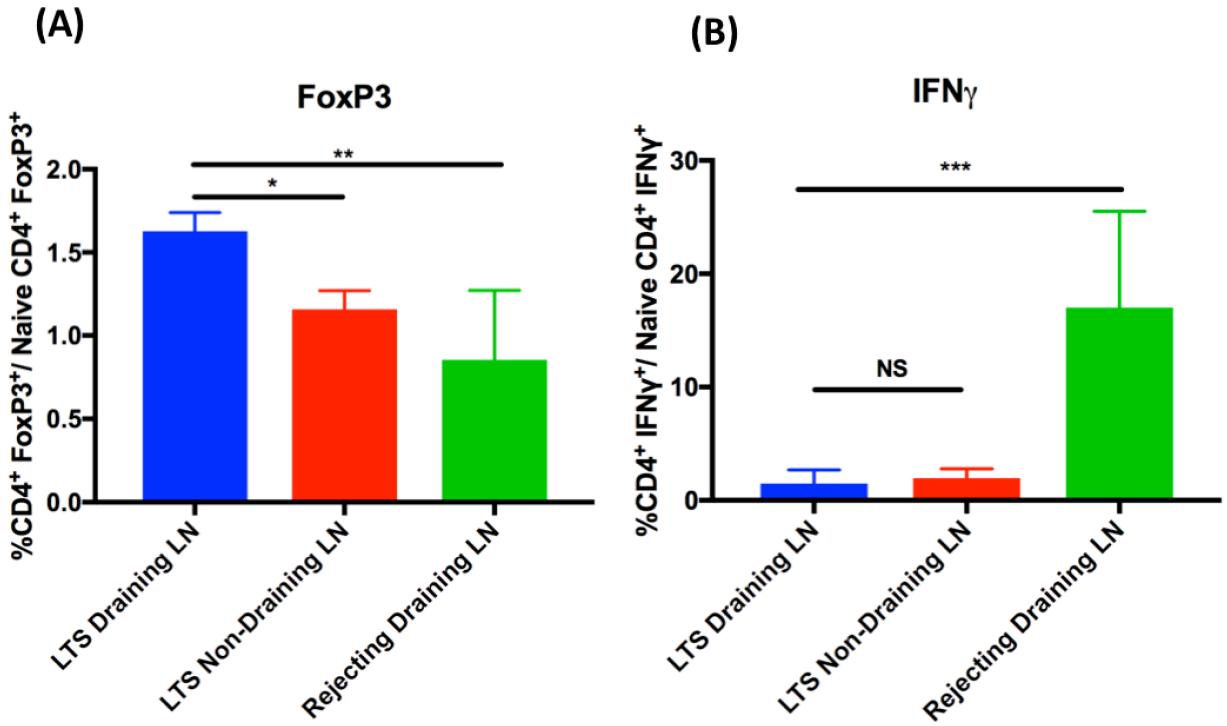

*Figure 10: Phenotypic analysis of CD4+ cells in draining and non-draining lymph nodes of long term surviving grafts treated with 50mg Recrutiment-MP (LTS, n=4) and draining lymph nodes of actively rejecting controls (n=3). Significant differences are indicated by * p < 0.05, ** p < 0.01, or *** p < 0.001.*

Tregs from Recruitment-MP treated animals exhibit superior Teff suppressive ability when compared to Tregs from naïve animals.

The suppressive and proliferative capacity of Treg and Teff isolated from Recruitment-MP treated VCA recipients and untreated naïve Lewis rats were measured in an *ex vivo* mixed lymphocyte reaction (MLR). Specifically, splenocytes from both Recruitment-MP treated VCA recipients and naïve animals were cell sorted into two groups: CD4+CD25hi (Tregs) or CD4+CD25- (Teff). Teff from both sets of animals were then cultured in a mixed lymphocyte reaction (MLR) with irradiated Brown Norway (donor) splenocytes. As shown in Figure 11A, there is no observed immune hypo-responsiveness with Teff isolated from Recruitment-MP treated animals when compared to naïve controls. To assess the suppressive function of Tregs from Recruitment-MP treated animals, Tregs from Naïve or Recruitment-MP treated animals were co-cultured with Teff from naïve Lewis (syngeneic) rats and irradiated Brown Norway (donor) splenocytes. Tregs isolated from Recruitment-MP treated animals were more effective than Naïve Treg at inhibiting proliferation of Naïve Teff stimulated with Brown Norway splenocytes ($p<0.05$) (Figure 11B, below).

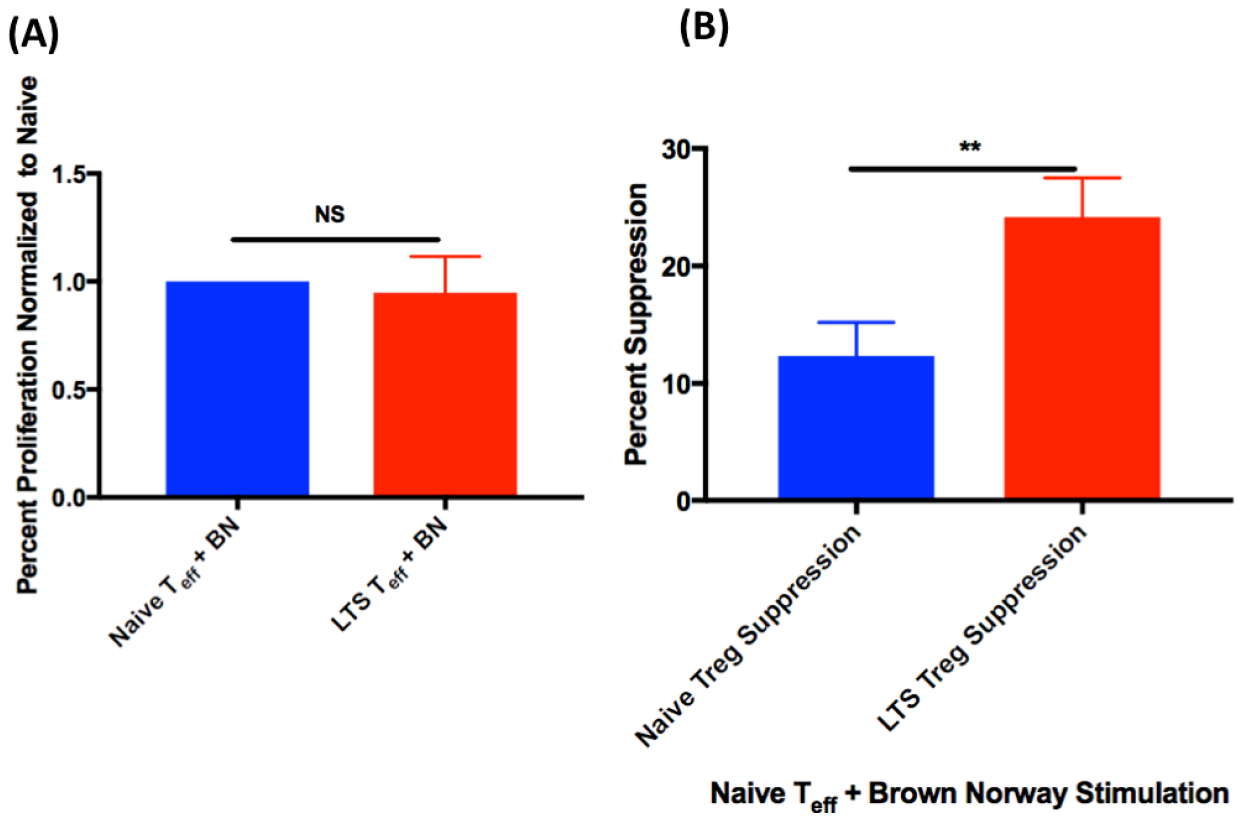

*Figure 11: Functional analysis of T cells isolated from animals with grafts treated with 50mg Recruitment-MP (LTS). There is no significant diference in proliferative capacity of $CD4^+CD25^-$ Teff (stimulated with Brown Norway (BN) splenoctyes) isolated from animals treated with Recruitment compared with $CD4^+CD25^-$ Teff (stimulated with BN splenoctyes) isolated from naïve Lewis rats (A). The percent proliferation was normalized to navie T_{eff} proliferation with BN stimluation. $CD4^+CD25^{hi}$ Tregs isolated from Recruitment-MP treated animals are more effecitve at suppressing BN mediated Naïve Teff proliferation than $CD4^+CD25^{hi}$ Tregs isolated from naïve Lewis rats (B). Significant differences are indicated by ** $p < 0.01$.*

Tregs from Recruitment-MP treated animals exhibit donor specific suppression of Teff proliferation.
In order to test the donor antigen specificity of CD4+CD25hi Tregs isolated from Recruitment-MP treated VCA recipients, yet another MLR was set up. Naïve Lewis CD4+CD25- Teff were co-cultured with CD4+CD25hi Tregs from Recruitment-MP treated VCA recipients and stimulated with either irradiated Brown Norway (donor) or Wistar Furth (third party, complete MHC mismatch) splenocytes. Tregs from long term surviving grafts showed enhanced suppressive function ($p<0.05$) against Brown Norway stimulation compared to Wistar Furth stimulation (Figure 12 A).

Recruitment-MP treatment confers systemic donor specific tolerance to hind-limb recipients in vivo.
To test whether Recruitment-MP is able to impart donor antigen specific tolerance *in vivo*, animals with long term surviving allografts (>200 days) were challenged with non-vascularized skin allografts from Lewis (syngeneic), Brown Norway (donor) and Wistar Furth (third party, complete MHC mismatch). After transplantation, all three grafts were sutured down, secured and protected with Xerofrm and gauze for 7 days to allow grafts to take. Further, it should be noted that these animals received no further immunosuppression or microparticle treatments beyond those previously noted at POD 21. All three animals actively rejected skin grafts from Wistar Furth animals, as evidenced by a lack of graft take, characterized by wound contraction and scarring. However, animals accepted both Lewis and Brown Norway grafts, with minimal wound contracture and eventual hair regrowth (Figure 12 B-C).

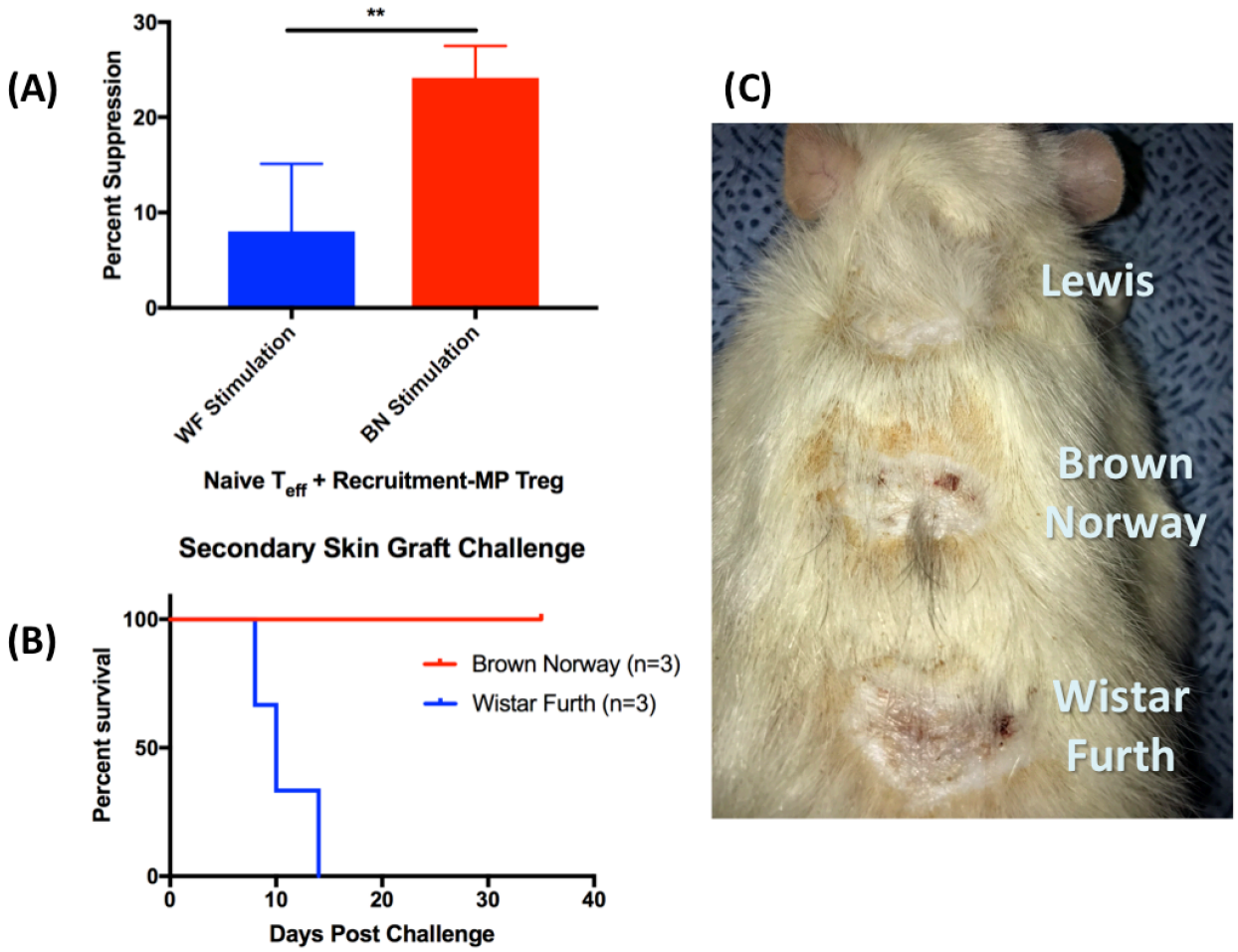

Figure 12. Recruitment-MP is able to confer donor antigen specific tolerance. CD4^{+}CD25^{hi} Tregs isolated from Recruitment-MP treated animals were co-cultured with CD4^{+}CD25^{-} Teff isolated from naïve Lewis rats and then subject to stimulation with either Brown Norway (BN) or Wistar Furth (WF) splenoctyes. Tregs isolated form Recruitment-MP treated animals are more effective at suppressing BN mediated proliferation than WF mediated proliferation (A). To demonstrate donor antigen specfic tolerance in vivo, Recrutiment-MP treated animals with long term surviving grafts were challenged with full thickenss non-vascularized skin grafts from BN and WF donors. 3/3 Recruitment-MP treated animals accepted BN grafts (as evidenced by wound healing and hair growth), while failing to accept WF grafts (as evidenced by contracture and graft necrosis) (B-C).

In the approach described above, a biomimetic strategy to locally recruit Tregs to a point sight *in vivo* was described. Specifically, it was demonstrated that CCL22 releasing microspheres (Recruitment-MP) are able to prolong rodent hind limb allograft survival and promote donor antigen specific tolerance. While this method has demonstrated great potential as a therapeutic in transplant immunology and other pathologies characterized by aberrant inflammation, it does have inherent limitations. Indeed, it is known that peripheral Tregs are particularly scarce, representing less than 2% of all Tregs in the human body. Accordingly, this strategy could prove to be problematic with respect to recruiting a large enough number of functional Tregs to be able to resolve inflammation, especially stringent models of inflammation such as VCA.

In this chapter an alternative approach for local enrichment of Tregs, with the goal of promoting transplant tolerance is put forth. Specifically, it was hypothesized that the *in vivo* induction of Tregs from naïve CD4+ T cells (a much more prevalent population of circulating lymphocytes) could represent an alternate way to employ a greater number of Treg at a local site. It is known that tolerogenic dendritic cells and even some cancerous tumors (as a means of evading immune recognition) are able to induce Tregs via secretion of Treg-trophic factors such as TGF-β, and IL-2 [175-179]. In addition, the avoidance of differentiation of naïve cells to TH17 lineages and maintenance of Treg suppressive phenotype is critically important[81], and the small molecule drug, rapamycin, has proven effective in achieving this *in vitro* and *in vivo[180]*. Based on this

information/inspiration, we have developed local, controlled release systems, herein referred to as **Tr**eg-Inducing (**TRI**) biodegradable systems that are able to controllably release extremely small amounts (nanograms/kilogram) of **T**GF-β, **R**apamycin and **I**L-2 (referred to as **TRI**-MP) to the local physiological milieu[127]. We have previously demonstrated that TRI-MP can promote *in vitro* differentiation of naïve cells to Treg, and can even reduce inflammation and abrogate symptoms in rodent models of dry eye disease and allergic contact dermatitis.

Herein, we put forth data that strongly suggests that TRI-MP can promote tolerance and indefinite allograft survival (>300 days) in an aggressive rat hind limb VCA model with complete MHC mismatch. Data suggest that TRI-MP can decrease intra graft expression of a panel of pro-inflammatory cytokines, increase the number of Tregs in graft draining lymphatics and promote donor antigen specific tolerance.

Methods – TRI-MP Study

Hind-Limb Transplantation

Using techniques developed in the University of Pittsburgh's Department of Plastic Surgery, hind limbs from donor Brown Norway Rats were transplanted to donor Lewis Rats. Specifically, donor femoral vessels were anastomosed end-to-end to recipient femoral vessels. Femoral osteosynthesis was performed with an 18-gauge intramedullary rod.

TRI-MP Fabrication and Characterization

IL-2 and TGF-β microparticles (IL2-MP and TGFβ-MP, respectively) were prepared using a well described double emulsion-evaporation technique (Figure 1). For the IL-2MP were fabricated as follows. 10μg of recombinant (r) mouse IL-2 (R&D Systems Minneapolis, MN) was mixed with 2 mg of BSA and 5 mM NaCl in 200 μl of de-ionized water. This solution was added to 4 ml of dichloromethane containing 200 mg of poly lactic-co-glycolic acid (PLGA; RG502H, Boehringer Ingelheim Chemicals Inc., Petersburg, VA), and the mixture was agitated using a sonicator (Vibra-Cell, Newton, CT) at 25% amplitude for 10 sec, creating the first water-oil emulsion. This emulsion was then mixed with 60 ml of 2% polyvinyl-alcohol (PVA, MW ~25,000, 98% hydrolyzed; Polysciences) under homogenization (L4RT-A, Silverson, procured through Fisher Scientific) at 3000 rpm for 1 min, creating the second emulsion. The resulting double-emulsion was then added to 80 ml of 1% PVA, and DCM was allowed to evaporate with the solution sitting on a magnetic stirrer at 600rpm for 3 hours. Subsequently, the microparticles were), washed 4 times in de-ionized water, and lyophilized (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 80 mTorr).

For TGFβ-MP the following conditions were used. 2μg of r-human TGF-β (CHO cell-derived, PeproTech, Rocky Hill, NJ) was mixed with 10 mg D-mannitol, 1 mg of BSA, and 15 mM NaCl in 200 μl of de-ionized water. This solution was added to 4 ml of dichloromethane containing 200 mg of PLGA (RG502H), and the mixture agitated using a sonicator at 25% amplitude for 10 sec, creating the first emulsion. This emulsion was then mixed with 60 ml of 2% PVA (containing 125 mM NaCl) under homogenization at 3000 rpm for 1 min, creating the second emulsion. The resulting double emulsion was then added to 80 ml of 1% PVA (containing 125 mM NaCl), and placed on a magnetic stir plate (600rpm) for 3 hours to allow DCM to evaporate.. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

Because Rapamycin is a lipophilic small molecule, Rapamycin-MP were prepared using the single emulsion-evaporation technique (Figure 13). Specifically, 1 mg of Rapamycin (LC labs, Woburn, MA) dissolved in DMSO was mixed with 4 ml of dichloromethane containing 200 mg of PLGA (RG502H). This solution was mixed with 60 ml of 2% PVA under homogenization at 3000 rpm for 1 min creating the microparticle emulsion. The resulting emulsion was then added to 80 ml of 1% PVA and placed on a magnetic stir plate (600rpm) for 3 hours to allow DCM to evaporate. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

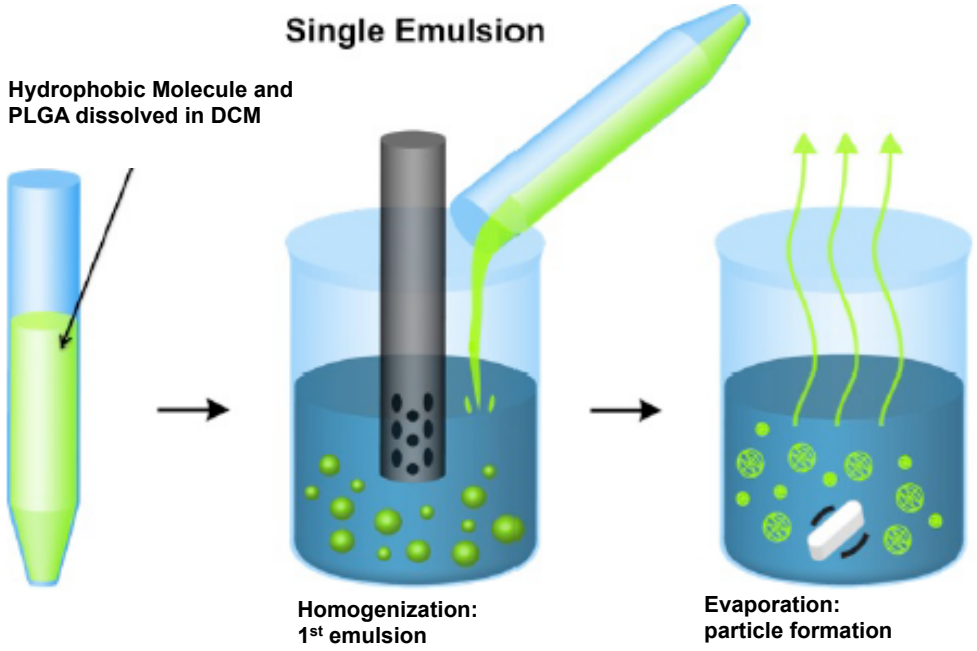

Figure 13: Illustration of the water-in-oil single emulsion technique used to fabricate Rapamycin micopartilces (Rapa-MP, a componenet of TRI-MP).

Release assays were completed as follows: 10 mg of IL-2 or TGFβ-MP were suspended in 1 ml of PBS with 1% BSA, and 10 mg of Rapamycin-MP was suspended in 1 ml of PBS (containing 0.2% Tween-80). Samples were then placed on an end-over-end roto-shaker at 37 °C. At daily time intervals, particle suspensions were centrifuged (250g, 5min), the supernatant removed, and the particles re-suspended in 1 ml of appropriate solution (Figure 2). The amount of either IL-2 or TGFβ in the supernatant was measured using a cytokine-specific ELISA (R&D systems, Minneapolis, MN), and the amount of Rapamycin was measured using spectrophotometry (absorbance at 278 nm). Surface characterization of microparticles was conducted using scanning electron microscopy (JEOL JSM-6510LV/LGS) and microparticle size distribution was determined by volume impedance measurements on a Beckman Coulter Counter (Multisizer-3, Beckman Coulter, Fullerton, CA).

Study Design and Groups

All hind-limb recipients in all groups received the same baseline immunosuppression protocol consisting of 21 days of FK506 (LC Laboratories, Woburn, MA) at a dose of 0.5 mg/kg, injected daily in (I.P.). Rats also received two doses of rabbit anti-rat lymphocyte serum (Accurate Chemical, Westbury NY) injected I.P. on POD -4 and 1 (Figure 3). Microparticles were injected subcutaneously in the lateral aspect transplanted limb (unless otherwise noted) at concentration of 10mg/ml. Animals receiving transplants were allocated into groups consisting of the following treatments, note all animals in all groups received the same baseline immunosuppression protocol. Animals receiving TRI-MP received 3mg of each microparticle formulation in 900µl sterile phosphate buffered saline (PBS). As controls animals also received injections of the individual components of TRI-MP (3mg of each formulation in 300µl PBS), the pairwise iterations of TRI-MP (TGFβ-MP + Rapa-MP, TGFβ-MP+ IL2-MP, IL2-MP + Rapa-MP, 3 mg of each formulation in 600µl PBS), TRI-MP injected in the contralateral (non-transplanted) limb, and Blank-MP (carrier).

Hind-Limb Allograft Monitoring
To assess rejection, hind limbs were monitored daily and scored for rejection (appearance grading) based on physical examination. Limbs 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 (Figure 4).

Histology
Skin and muscle samples were obtained from the transplanted limbs of animals at their experimental endpoint (progressive grade III rejection or long term survival >200days). Samples were fixed in 10% neutral buffed formalin, paraffin embedded, sectioned into 5μm slices and stained hematoxylin and eosin (H&E) for microscopic examination of tissue architecture and mononuclear infiltration.

Flow Cytometric Analysis
Draining and non-draining lymph nodes (DLN and NDLN) were harvested at their experimental endpoint (progressive grade III rejection or long term survival >200days). Lymph node samples were then mechanically processed and strained through a 70μm filter to form a single cell suspension. Cells were stained with the following fluorescently labeled antibodies: anti-CD4 PE , anti-CD25 PerCP/Cy5.5, anti-FoxP3 Pacific Blue and anti-IFNγ APC (eBioscience, San Diego, CA). For intracellular cytokine staining, the cells were placed in a 96-well plate overnight in cell culture media with Cell Stimulation Cocktail (plus protein transport inhibitor, eBioscience) and stained with anti-IFN-γ. Stained cells were then analyzed using FlowJO (Ashland, OR).

Gene Expression
Gene expression profiles of inflammatory markers were evaluated in the skin and lymph nodes of long term survivors, actively rejecting and naïve (self) rats. Total RNA was extracted from samples 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 (Rn00594078_m1 Dye: VIC-MGB_PL), TNF (Rn99999017_m1 Dye: VIC-MGB_PL), Perforin-1 (Rn00569095_m1 Dye: VIC-MGB_PL), Serglycin (Rn00571605_m1 Dye: VIC-MGB_PL), IL-17 (Rn01757168_m1 Dye: VIC-MGB_PL) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, endogenous control, Rn99999916_s1). Duplex reactions (target gene + GAPDH) were run and analyzed on a StepOnePlus Real-Time PCR System. Relative fold changes of IFNγ, TNF, Perforin-1, Serglycin, and IL-17 expression were calculated and normalized based on the $2^{-\Delta\Delta Ct}$ method and then further normalized to naïve tissue, skin biopsies from naïve animals or contralateral limbs serving as untreated controls.

Cell Proliferation and Suppression Assays
Spleens from rats with long-term surviving hind limbs and naïve rats were processed into a single cell suspension. Red blood cells (RBC) were lysed using RBC lysis buffer (Thermo Fisher Scientific, Pittsburgh PA). $CD4^+$ T cells were isolated by CD4 T cell enrichment columns according to the manufacturer's instructions (Milyteni Biotech, Auburn CA). $CD4^+$ enriched cells were then stained with anti-CD4 PE and anti-CD25 perCP/Cy5.5. $CD4^+CD25^{low}$ (Teff) and $CD4^+CD25^{hi}$ (Treg) populations were sorted using a fluorescence-activated cell sorter. To assess proliferative function, $CD4^+CD25^{low}$ (Teff) from long-term surviving and naïve rats were stained with VPD45 (BD Biosciences, San Jose, CA) and each was co-cultured/stimulated with irradiated splenocytes harvested from Brown Norway (BN) rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using FlowJO (Ashland, OR). The proliferative capacity of Teff from long term surviving rats was normalized to that of naïve rats.

To quantify suppressive cell function $CD4^+CD25^{hi}$ (Treg) isolated from long term surviving and naïve rats were tested for their ability to suppress Teff proliferation in an MLR. $CD4^+CD25^{low}$ Teff from naïve rats were stained with VPD45 and co-cultured/stimulated with irradiated splenocytes harvested from Brown Norway (BN) rats and $CD4^+CD25^{hi}$ Tregs harvested from either long term surviving or naïve rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using FlowJO (Ashland, OR). Percent suppression was calculated using the formula:

$$Percent\ Suppression = 1 - \left(\frac{Percent\ Proliferation\ Naive\ Teff + BN\ Splenoctye + Treg}{Percent\ Proliferation\ of\ Naive\ Teff + BN\ Splenocyte}\right)$$

A final MLR was set up to test for antigen specificity in $CD4^+CD25^{hi}$ (Treg) isolated from long term surviving rats. $CD4^+CD25^{low}$ Teff from naïve rats were stained with VPD450 and stimulated with either Brown Norway (BN) or Wistar Furth (WF) irradiated splenocytes and then co-cultured with $CD4^+CD25^{hi}$ Tregs isolated from long term surviving rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using FlowJO (Ashland, OR). Percent suppression was calculated using the formula:

$$Percent\ Suppression = 1 - \left(\frac{Percent\ Proliferation\ Naive\ Teff + BN/WF\ Splenoctye + Treg}{Percent\ Proliferation\ of\ Naive\ Teff + BN/WF\ Splenocyte}\right)$$

Full Thickness, Nonvascularized Secondary Skin Grafting

Donor antigen specific tolerance was assessed *in vivo* in three long-term surviving animals from the 50mg Recruitment MP group via secondary skin graft challenge. Skin allografts were harvested from donor strain (BN) or third-party strain rats (WF) and transplanted to the long-term survivors >200 days after VCA (Figure 7). Grafts were bolstered in place for 5 days and subsequently evaluated daily for signs of rejection. Rejection was defined as necrosis of the skin graft.

Statistics

All data was expressed as mean ± standard deviation, followed by Student's t-test for two independent samples or analysis of one-way analysis of variance. The influence of various treatments on VCA survival was analyzed using a log-rank test. $p < 0.05$ was considered significant.

Results

Characterization of TRI-MP

IL2-MP, TGFβ-MP, and Rapa-MP were all prepared under similar conditions, using the same polymer (RG502H, viscosity 0.16-0.24 dl/g). Scanning electron micrographs (Figure 14B, middle panel) show that individual particles are spherical and confirm the volume average size distributions (IL2MP = 17.2 μm; TGFβMP = 16.7 ± 6.3 μm; rapaMP = 15.7 μm). Additionally, the images show that IL2MP have slightly porous exterior surfaces. These particles were specifically formulated to be porous (by altering osmotic pressures between the inner emulsion and the outside aqueous phase during microparticle preparation) so that a high initial burst followed by continuous release could be obtained (Figure 14C). Further, we observe a linear release of TGF-β following a ~2 week lag phase, and a continuous release from Rapa-MP (Figure 14C, bottom panel).

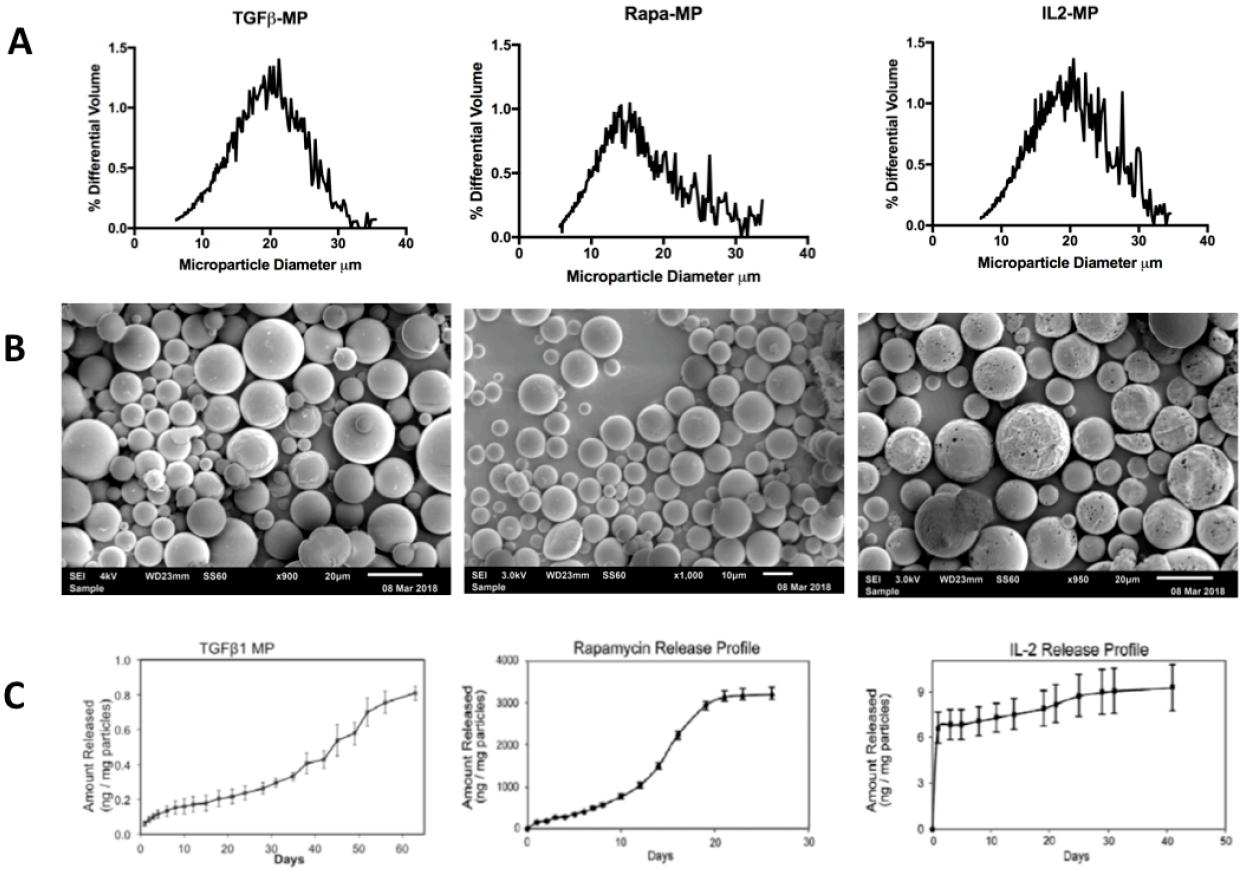

Figure 14. Characterization of TRI-MP. (A) Size distribution of TGFβ-MP, Rapa-MP and IL2-MP. (B) Representative SEM images of TGFβ-MP, Rapa-MP and IL2-MP. (C) Release kinetics of TGFβ-MP, Rapa-MP and IL2-MP (n=3 for each formulation).

Subcutaneous, intragraft treatment with TRI-MP is able to prolong rodent hind-limb transplant survival indefinitely.

To investigate the ability of TRI-MP to prevent allorecognition and promote long-term survival in VCA, we employed a rodent hind-limb allotransplant model. Specifically, hind limbs were transplanted from Brown Norway (BN) donors to Lewis (LEW) recipients by an experienced microsurgeon. These strains were chosen as they represent a complete MHC mismatch. All animals receiving transplants received a short course baseline immunosuppression protocol consisting of 0.05 mg/kg FK506 as well as two 0.5cc doses of rabbit anti-rat lymphocyte serum (ALS). As shown in Figure 17B, grafts only receiving this baseline immunosuppression protocol reliably experience rejection 2-3 weeks after discontinuing systemic FK506. However 11/12 grafts receiving the same baseline immunosuppression (in addition to two subcutaneous injections of TRI-MP) go on to survive indefinitely (>300 days). Notably, individual components of TRI-MP (TGFβ-MP, Rapamycin-MP, and IL-2MP) are also not able to confer reliable long-term survival to hind limb recipients (Figure 15B). Moreover, administration of all pairwise iterations of TRI-MP did not prolong hind-limb survival when compared to animals only receiving the baseline immunosuppression treatment. As a control, we observed that TRI-MP treatment in the contralateral (non-transplanted) hind limb was not able to yield long-term survival, suggesting that local release is required for formulations to be effective (Figure 15C).

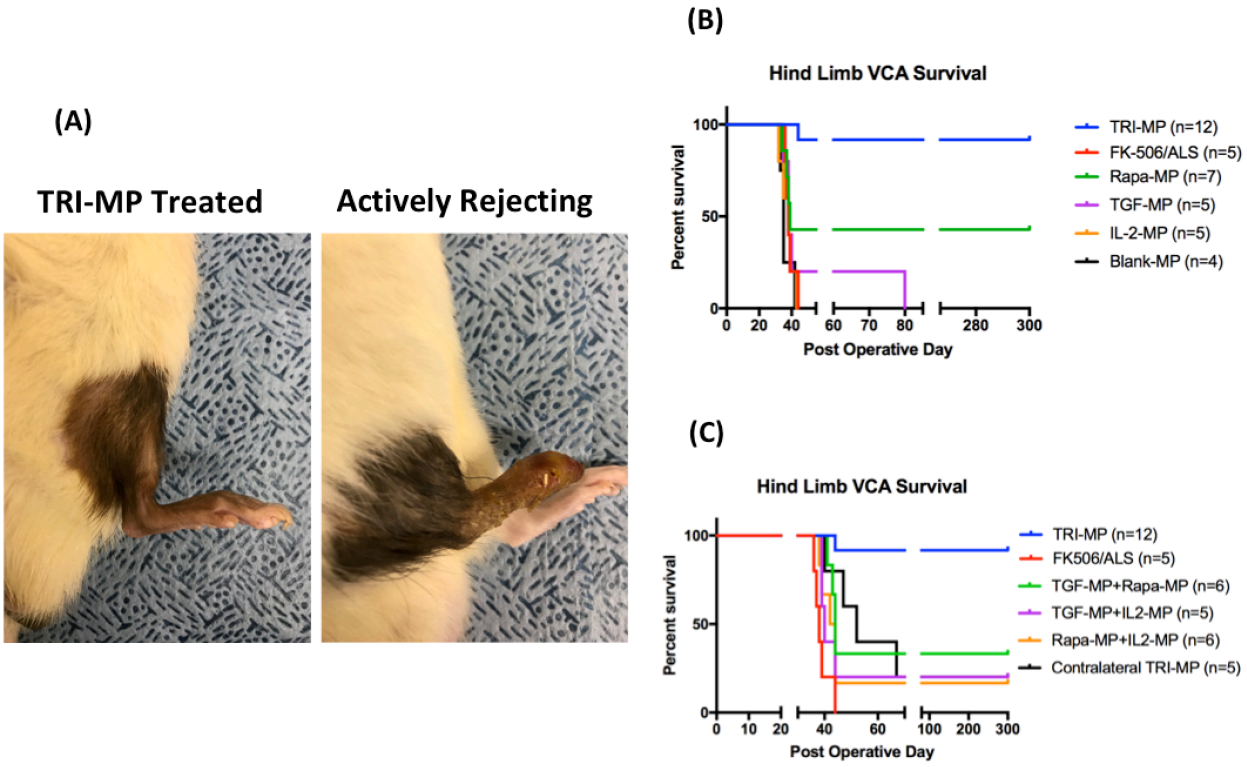

Figure 15. TRI-MP can prevent rejection and promote long term survival in rodent hind limb recipients. (A) Reprsentitive images of TRI-MP treated hind limb (POD>300) showing no signs of rejection and an actively rejecting control. (B) TRI-MP is able to prolong hind limb survival (>300 days in 11/12 anaimals) in the abscense of long term systemic immunosuppression (p<0.001 for TRI-MP vs. baseline immunosuppression) . The individual components alone (TGFβ-MP, Rapa-MP and IL2-MP) are not able to confer reliable long term survival (p<0.05 for TRI-MP vs. Rapa-MP, p<0001 for TRI-MP vs .all other individual components). (C) Pairwise iterations of TRI-MP, as well as treatment with TRI-MP in the contralateral (non-transplanted) limb were also unable to prolong hindlimb survivial (p<0.001 for TRI-MP vs. all pairwise controls).

Microscopically, skin samples taken from TRI-MP treated grafts demonstrate an intact dermis and epidermis and show limited mononuclear infiltration and tissue architecture similar to that seen in naïve skin samples (Figure 18). Muscle samples also exhibit normal tissue architecture and limited cellular infiltration. Skin and muscle biopsies taken from actively rejecting animals show dense mononuclear infiltration and significant destruction of tissue architecture, mirroring what was observed macroscopically (Figure 16, below).

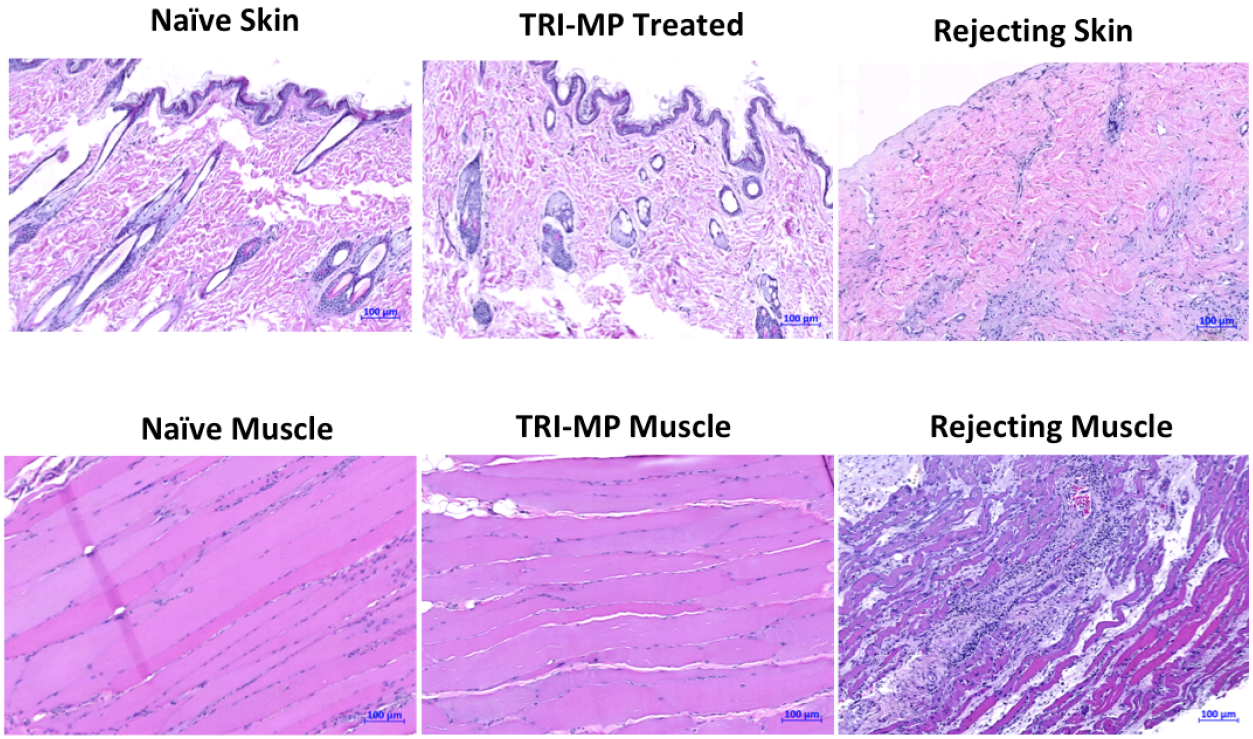

Figure 16. TRI-MP is able to preserve the architectural integrity of intra-graft tissues (muscle and skin). Tissue samples taken from rejecing animals display complete destruction of tissue archtecture with dense mononuclear infiltration (right pannel).

Local expression of pro-inflammatory cytokines is decreased in tissue samples from TRI-MP treated animals.

Expression of a panel of pro-inflammatory cytokines was examined in skin and lymph node biopsies taken from TRI-MP treated grafts, actively rejecting grafts and naïve animals. Figure 17 demonstrates that both intragraft skin biopsies as well as draining lymph nodes from TRI-MP treated long term surviving grafts exhibit significantly lower expression of TNF, IL-17, Serglycin, Perforin-1 and IFN compared to skin and draining lymph node samples taken from actively rejecting controls.

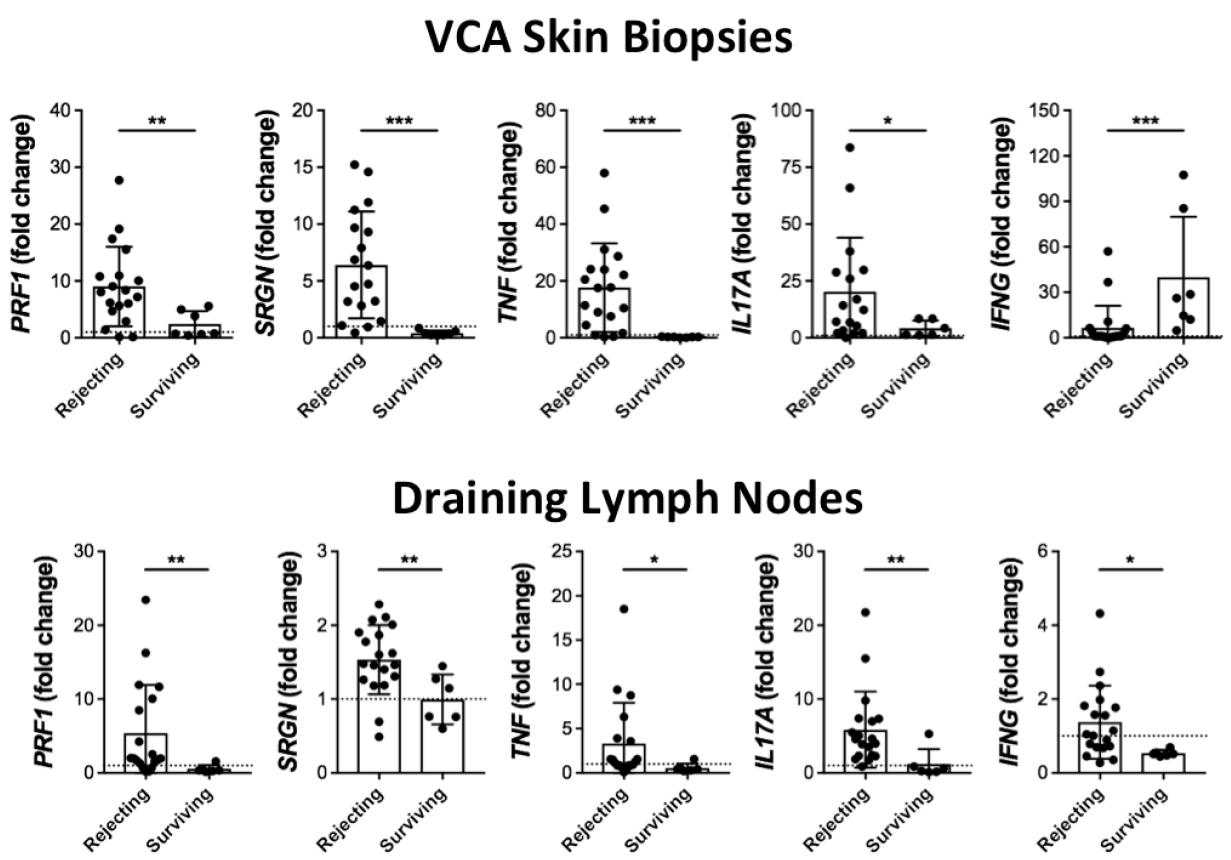

*Figure 17. Relative mRNA expression in skin samples and draining lymph nodes from rejecting hind limb allografts (n=17-20 for skin, n=19 for draining lymph nodes) vs. surviving TRI-MP treated hind limb allografts (n=7 for skin and n=6 for lymph nodes). Expression levels are presented as fold changes ($2^{-\Delta\Delta Ct}$) relative to naïve skin (N=15-19). Bars represent mean ± SD, and dots represent values from individual rats. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.*

TRI-MP increases the percentage of FoxP3+ Tregs and decreases the percentage of IFNγ$^+$CD4$^+$ cells in the draining lymph nodes of TRI-MP treated animals.

In order to analyze potential trends in the phenotype of local CD4+ helper T cells in TRI-MP treated animals and actively rejecting controls, draining inguinal lymph nodes were harvested and stained for markers of Tregs (FoxP3) and T effectors (IFNγ). Figure 18A illustrates that (when normalized to naïve lymph nodes) percentages of CD4+CD25hiFoxP3+ cells are observed to be significantly higher in the draining lymph nodes of TRI-MP treated animals compared to actively rejecting controls. Conversely, percentages of CD4+IFNγ+ cells were significantly higher in draining lymph nodes of actively rejecting animals when compared to TRI-MP treated animals (Figure 18B).

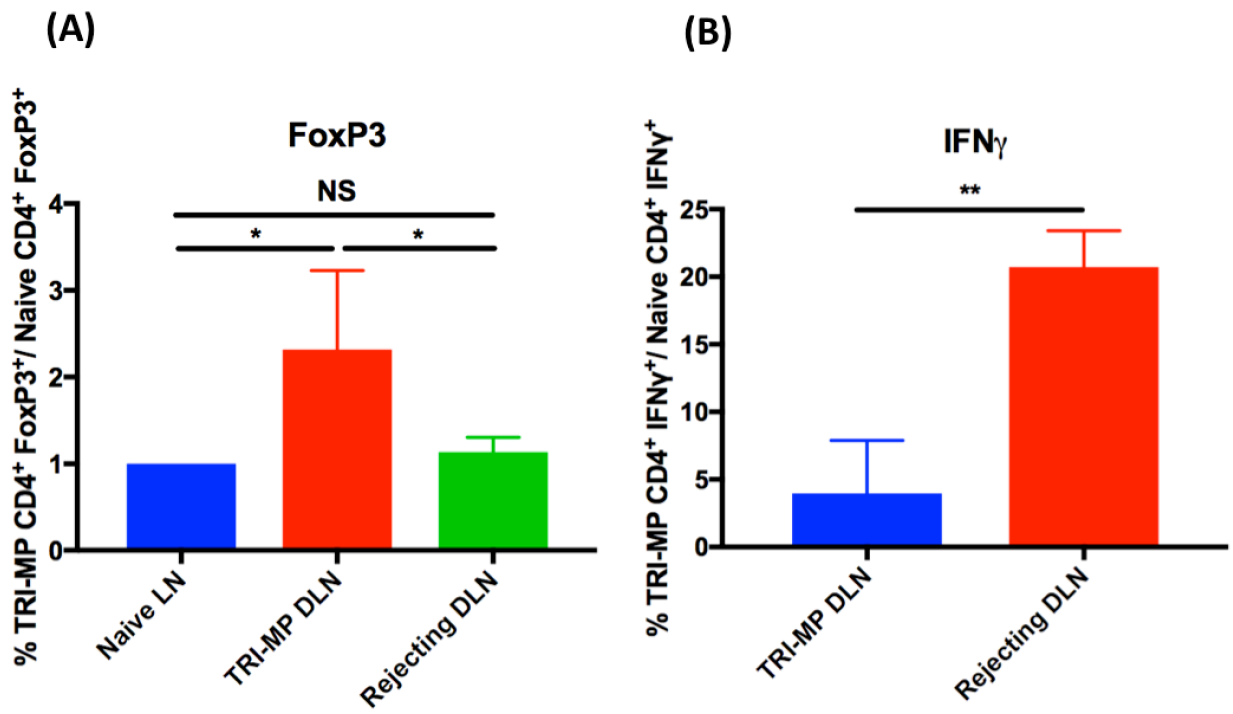

*Figure 18. Phenotypic analysis of CD4+ cells in draining and non-draining lymph nodes of TRI-MP treated long term surviving grafts (LTS, n=4) and draining lymph nodes of actively rejecting controls (n=3). Percentages of $CD4^+FoxP3^+$ and $CD4+IFN\gamma^+$ cells in TRI-MP treated and Rejecting animals were normalized to percentages of $CD4^+FoxP3^+$ and $CD4+IFN\gamma^+$ cells in naïve Lewis rats. Significant differences are indicated by * $p < 0.05$ or ** $p < 0.01$.*

Effector T cells (Teff) isolated form TRI-MP treated animals do not exhibit immune-hyporesponsiveness. CD4+ T cells were isolated from splenocytes of TRI-MP treated rats as well as naïve rats. These CD4+ cells were further sorted into Treg (CD4+CD25hi) and Teff (CD4+CD25-) populations. As shown in figure 19A Teff isolated from TRI-MP do not appear to possess any immune hypo-responsiveness (when normalized to naïve Teff) when stimulated with irradiated Brown Norway (donor) splenocytes.

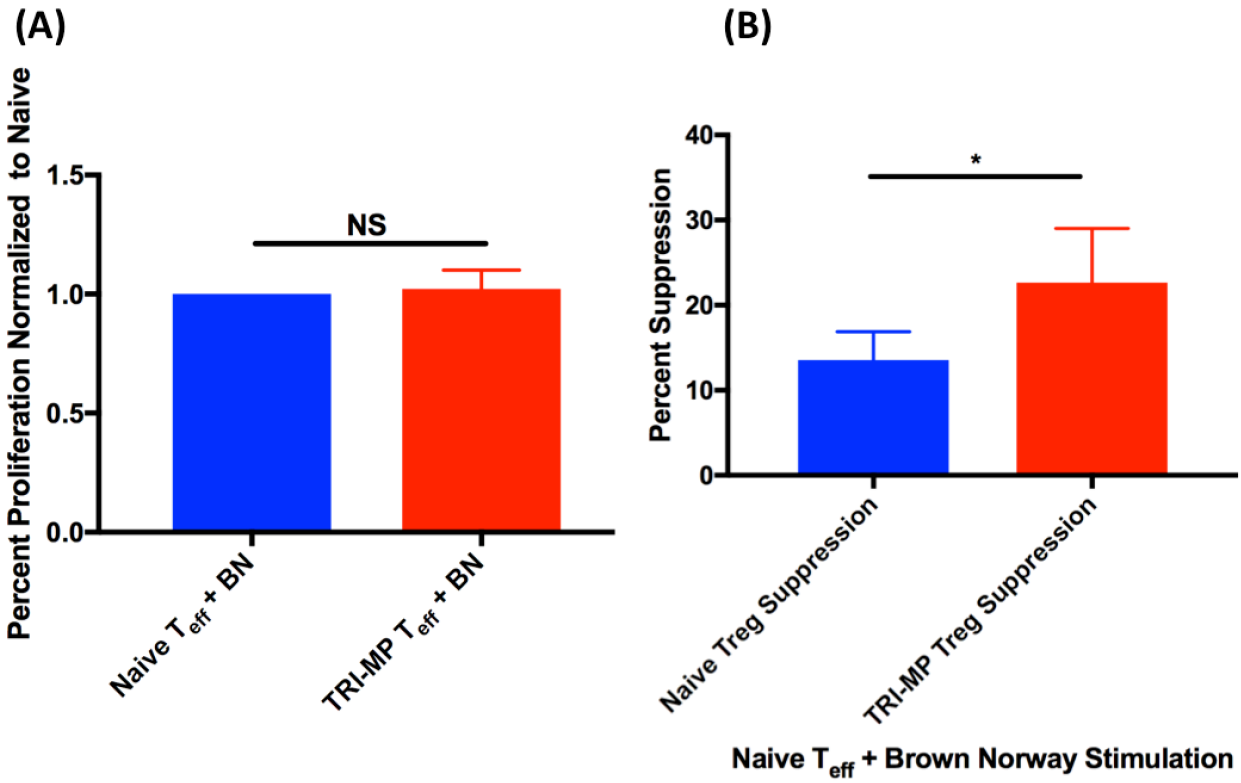

*Figure 19. Functional analysis of T cells isolated from animals with TRI-MP treated grafts. (A) There is no significant diference in proliferative capacity of $CD4^+CD25^-$ Teff (stimulated with Brown Norway (BN) splenoctyes) isolated from animals treated with TRI-MP compared with $CD4^+CD25^-$ Teff (stimulated with BN splenoctyes) isolated from naïve Lewis rats. The percent proliferation was normalized to navie T_{eff} proliferation with BN stimluation. (B) $CD4^+CD25^{hi}$ Tregs isolated from TRI-MP treated animals are more effecitve at suppressing BN mediated Naïve Teff proliferation than $CD4^+CD25^{hi}$ Tregs isolated from naïve Lewis rats. Significant differences are indicated by * $p < 0.05$*

CD4+CD25+ Tregs isolated from TRI-MP treated animals exhibit superior suppressive function and donor antigen specificity.

To assess functional abilities, CD4+CD25hi Tregs were isolated from splenocytes of TRI-MP treated rats with long term surviving hind-limbs as well as from naïve Lewis rats. These two sets of Tregs were then co-cultured Naïve CD4+CD25- Teff stimulated with irradiated Brown Norway (Donor) splenocytes. Figure 19B (above) demonstrates that Tregs isolated from TRI-MP treated animals are more effective at suppressing Brown Norway stimulation than Tregs isolated from naïve rats.

In a similar, parallel experiment, CD4+CD25hi Tregs isolated from TRI-MP treated rats were tested antigen specificity. Specifically, Tregs from TRI-MP treated animals were co-cultured with CD4+CD25 Teff from naïve rats stimulated with either irradiated Brown Norway (donor) or Wistar Furth (third party) splenocytes. Indeed, data represented Figure 20A suggests that in addition to possessing superior suppressive function when compared to naïve Tregs, Tregs from TRI-MP treated animals also appear to be more effective at suppressing Brown Norway mediated stimulation.

TRI-MP is able to promote donor antigen specific tolerance in vivo.
Antigen specific tolerance was also tested *in vivo*. Specifically, TRI-MP recipients with long term surviving grafts (>200 days, n=3) were subject to secondary, non-vascularized skin grafting from both Brown Norway (donor) and Wistar Furth (third party) recipients. As shown in Figure 20B-C, long term survivors accept Brown Norway grafts, as evidenced by healing skin and hair growth while failing to accept Wistar Furth skin grafts, as evidenced by graft necrosis and contracture.

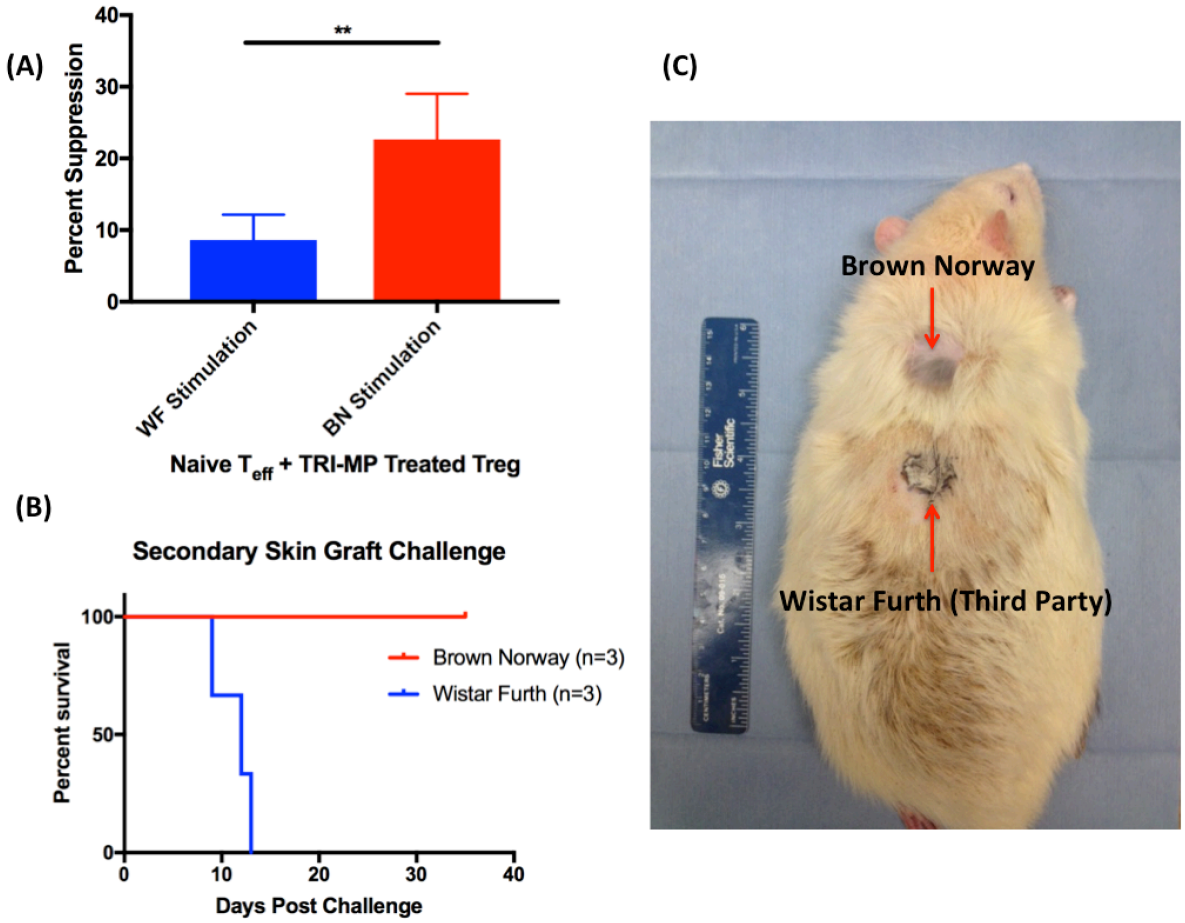

Figure 2. TRI-MP imparts antigen specfic toelrance toward rat hind libm recipients. (A) CD4^{+}CD25^{hi} Tregs isolated from TRI-MP treated animals were co-cultured with CD4^{+}CD25^{-} Teff isolated from naïve Lewis rats and then subject to stimulation with either Brown Norway (BN) or Wistar Furth (WF) splenoctyes. Tregs isolated form TRI-MP treated animals are more effective at suppressing BN mediated proliferation than WF mediated proliferation. To demonstrate donor antigen specfic tolerance in vivo, TRI-MP treated animals with long term surviving grafts were challenged with full thickenss non-vascularized skin grafts from BN and WF donors. (B-C) TRI-MP treated animals (n=3) accepted BN grafts (as evidenced by wound healing and hair growth), while failing to accept WF grafts (as evidenced by contracture and graft necrosis).

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, which will take place during the NCE, our group will complete and wrap up the large animal (swine) portion of this work. Since Dr. Gorantla's transition to Wake, there have been delays outside of our control involving personnel issues in the Department of Plastic Surgery, University of Pittsburgh. This project was originally proposed with the Department of Plastic Surgery providing the surgical support for the large animal model as Dr. Little did not have the expertise or infra structure to complete these studies in the Department of Chemical Engineering. However, the current situation with personnel transition in the Plastic Surgery lab make it very difficult for us to realistically complete the milestones and deliverables for the final phase of the porcine study. Thus, the large animal study will be performed at Wake Forest University under Dr. Gorantla's supervision. Dr.Gorantla currently submitted the protocol for IACUC approval at the Wake Forest University. Following the approval by Wake Forest University IACUC, we will submit for ACURO approval.

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: We have written two manuscripts detailing the promosing results obtained from our rodent studies examining the effects of Recruitment-MP and TRI-MP on VCA survival and tolerance. The first manuscript, CCL22 part, was accepted by PNAS. The TRI-MP part is currently under evaluation in Science Advances. We are hopeful that it will be accepted. The current (preliminary) citations are below.

1. Fisher JD, Wensheng Z, Balmert SC, Aral AM, Acharya AP, **Kulahci Y**, Jingjing Li, Turnquist HR, Thomson AW, Solari MG, Gorantla VS, Little SR. In Situ Recruitment of Regulatory T Cells Promotes Donor-Specific Tolerance in Vascularized Composite Allotransplantation. Science Advances. (Revision submitted- October 2, 2019)
2. Fisher JD, Balmert SC, Wensheng Z, Schweizer R, Schnider J, Komatsu C, Dong L, Erbas VE, Unadkat JV, Aral AM, Acharya AP, **Kulahci Y**, Turnquist HR, Thomson AW, Solari MG, Gorantla VS, Little SR. Treg Inducing Microparticles Promote Donor-Specific Tolerance in Experimental Vascularized Composite Allotransplantation. Proc Natl Acad Sci U S A. (Accepted- October 30, 2019)

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	3
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 immunology.

Name:	Mario Solari
Project Role	Microsurgeon
Researcher Identifier	
Nearest person month worked	1.2
Contribution to Project	Dr. Solari is responsible for large animal experimental design, trouble shooting, and performing large animal surgery

Name:	Yalcin Kulahci
Project Role	Postdoctoral Fellow
Researcher Identifier	
Nearest person month worked	6
Contribution to Project	Dr. Kulahci is responsible for experimental design for this project and trouble shooting especially with respect to animal CTA and immunobiology and performing microsurgery.

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:	Ethan Bassin
Project Role	Graduate Student
Researcher Identifier	
Nearest person month worked	12
Contribution to Project	Mr. Bassin is a graduate student responsible for fabrication of microparticles and conducting immunoassays.
Funding Support	DoD Award W81XWH-15-1-0244

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.