REGULATORY T CELL ENRICHING MICROPARTICLES FOR PROMOTING TOLERANCE IN VASCULARIZED COMPOSITE ALLOTRANSPLANTATION

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Vascularized Composite Allotransplantation (VCA) is an emerging field encompassing transplantation of limbs and face. In clinical VCA, combination therapy with two or more immunosuppressive drugs is standard. Nonetheless, side effects associated with the administration of lifelong, high dose, multidrug immunosuppression continue to hamper wider implementation of VCA. Greater feasibility, wider acceptability and routine applicability of VCA will only be realized if the risks of transplant rejection and chronic immunosuppression are minimized. An immediate goal is the exploration of novel strategies that achieve donor-specific immune hypo-responsiveness via local immunomodulation, minimizing or eliminating the need for systemic immunosuppression.

Interestingly, the cells of our bodies have evolved to utilize a host of strategies to maintain immunological homeostasis, representing a level of sophistication that dwarfs current attempts at immunosuppression. As a hallmark example, our bodies contain a subset of lymphocytes called regulatory T cells (Treg) that play a critical role in establishing and maintaining immunological homeostasis. However, because Tregs are found in low numbers throughout the body, strategies to harness them for therapeutic use have thus far focused on *ex vivo* expansion followed by *in vivo*

re-administration. Though promising, the clinical implementation of these approaches is faced with numerous logistical and regulatory hurdles.

To this end, our group has developed synthetic approaches utilizing cell sized, biocompatible, biodegradable microspheres composed of poly(lactide-co-glycolide) acid (PLGA) capable of releasing factors that can locally enrich for Treg. Specifically, PLGA microparticles that release the Treg recruiting chemokine CCL22 (referred to as Recruitment-MP) were fabricated and tested for their ability to prevent allograft rejection in a rodent model of hindlimb transplantation. Indeed, Recruitment-MP was able to prolong hind limb survival indefinitely and promote donor antigen-specific tolerance.

Finally, an alternative strategy to locally enrich for Tregs via their induction from Naïve T cells was described. **Tr**eg **I**nducing microparticles (**TRI**-MP) that release **T**GF-β, **R**apamycin, and **I**L-2, were also able to promote long-term graft survival and (importantly) induce donor-specific tolerance. Taken together, the Treg enriching formulations described herein are synthetic systems that take inspiration from nature's mechanisms for resolving inflammation and have the potential to prevent aberrant inflammation in the context of allotransplantation.

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1.0 INTRODUCTION

The concept of replacing or transplanting damaged organs and tissues has likely existed as long as human disease and trauma. Initially only described as the medicine of mythology, the first "descriptions" of transplantation come from legends of a Chinese physician Pien Chi'ao conducting a double heart transplant in 5th century B.C. between two men who suffered from an imbalance of heart and spirit. Further descriptions came from Roman Catholic reports of Saints Cosmas and Damian who were able to transfer a limb from a diseased Ethiopian soldier to replace the amputated leg of an esteemed churchman [1-3]. The most likely accounts of early transplants can be dated to 2nd century B.C. when Indian surgeon Sushruta would employ autografted skin for nose reconstruction. Later, Italian surgeon Gasparo Tagliacozzi observed that he was able to conduct successful skin autografts, but experienced failure when using allografts [4].

Nonetheless, it was not until WWI that the modern field of transplantation (and transplant immunology) began to make significant strides. The most notable advances came from the laboratory of Dr. Peter Medawar, who conducted basic science research studying skin allograft immunology and tolerance (this would ultimately result in a Nobel Prize for Medwar). However, the first successful transplant would not occur until 1954, when Dr. Joseph Murray was able to transplant a kidney between identical twins [1]. The work conducted by Dr. Murray led to attempts to transplant other types of solid organs (including lung, liver, and additional kidney transplants),

however acute rejection and side effects from early/rudimentary immunosuppression prevented any long-term success for these early patients.

It was not until the 1970s that effective combination immunosuppression was able to yield successful long-term survival in early kidney transplant recipients [1-6]. Further advances including the discovery of calcineurin inhibitor drugs (FK506 and Cyclosporin), the development of medical devices (such as the heart-lung machine), improvement in microsurgical techniques and a more mature understanding of the immune system has made the discipline of transplantation a ubiquitous and life-saving therapeutic strategy [4]. Despite the significant advances made in science and medicine in the past 100 years, allorecognition and rejection remain the two largest hurdles facing the field of allotransplantation.

1.1 THE DEVELOPMENT AND CLINICAL IMPLEMENTATION OF MODERN IMMUNOSUPPRESSION

The success of organ transplantation to date has hinged on the continued development (and improvement) of immunosuppression. Though somewhat subjective, the development of immunosuppression can be divided into four distinct eras Figure 1. Early immunosuppression from 1954 to 1984 employed rather rudimentary attempts at immunosuppression including total body irradiation, splenectomy, thymectomy, and high dose corticosteroids [7]. More eloquent developments, including the antiproliferative agent Azathioprine and the T cell depleting polyclonal antibody Anti Lymphocyte Globulin (ALG) yielded success (in the context of kidney transplantation) in the late 1960s and 1970s as part of Thomas Starzl's highly successful "triple cocktail" consisting of Azathioprine, ALG and Prednisone [7].

The next era spanning from 1984 to 1995 included the development of the calcineurin inhibitor Cyclosporin (and later FK506) as well as the T cell monoclonal antibody OKT-3 which would eventually replace ALG [4-6]. Further, the development of the antimetabolite mycophenolic acid would replace azathioprine [5, 6].

From 1995 onward the field of transplant immunology would see the development of a monoclonal antibody (Basiliximab) to the alpha chain of the IL-2 receptor on T cells as well as the mTOR inhibitor Rapamycin [6]. The fourth era of immunosuppression will theoretically encompass current experimental therapies being reported in small trials and preclinical studies in the literature. This will include the use of ex vivo cell therapy, organ engineering, and smart immunosuppression using biomaterials and drug delivery systems such as micro- and nanoparticles (MNP) [8-14].

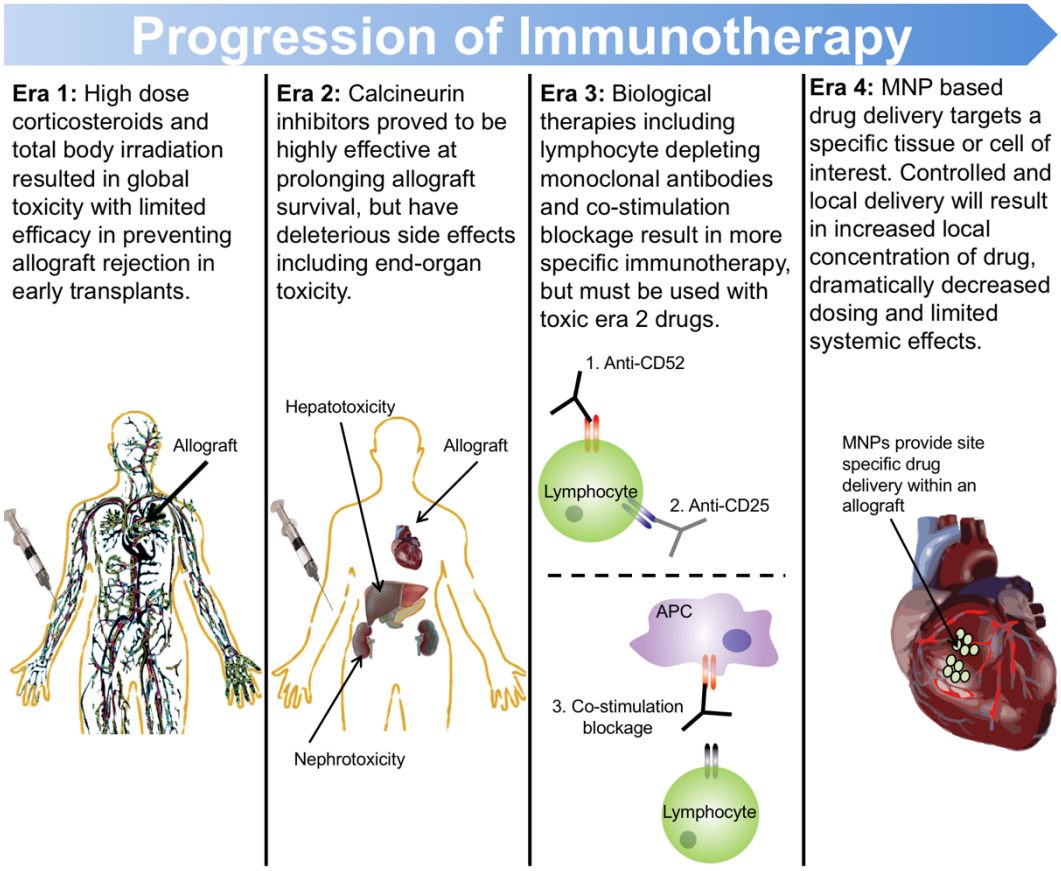

Figure 1. The Development and clinical implementation of immunosuppression through four distinct eras.

Initial immunosuppression protocols employed rudimentary and highly toxic techniques (such as whole-body irradiation) that yielded poor outcomes. The development more powerful and specific drugs (such as FK506) resulted in the widespread implementation of solid organ transplantation as a solution to organ dysfunction. It is likely that future therapies will utilize drug delivery systems to allow for targeted delivery to the graft. Adapted from Fisher, J.D. et. al., 2015 [14].

1.2 MAINTENANCE THERAPY

Maintenance immunosuppression therapy is currently the long-term solution to staving off acute allograft rejection and dysfunction [4-6, 15, 16]. The goal of maintenance therapy is to use

multiple pharmacological agents that affect different immunological targets. This approach aims to achieve efficient immunosuppression while simultaneously minimizing side effects associated with individual drugs. The risk of acute rejection usually is highest within the first three months following transplantation. Therefore, higher doses of maintenance therapy are employed in this critical period and subsequently weaned to lower (less toxic) doses [4-6]. The most commonly used maintenance therapy are 1.) corticosteroids, 2.) antiproliferative agents 3.) calcineurin inhibitors (CNIs), 4.) mammalian target of rapamycin (mTOR) inhibitors, 5.) costimulation blockers [4-6]. The combination and choice of maintenance therapy agents used can vary based on multiple factors such as the type of transplant, health center protocols, patient comorbidities and physician preference [4-6].

1.2.1 Corticosteroids

Corticosteroids are naturally occurring hormones in nature with many physiological functions, including immunosuppression. Synthetic derivatives of corticosteroids (such as prednisone) that have longer half-lives, greater stability, and decreased toxicity are the most common and potent drugs for suppressing inflammation not only in the context of transplantation but other conditions characterized by aberrant inflammation (including autoimmune disorders) [17, 18].

Generally speaking, corticosteroids have been the mainstay of immunosuppression for various pathologies since the 1920s. Mechanistically, corticosteroids can modulate the immune system at multiple checkpoints due to their ability to agonize the glucocorticoid receptor (GR), which is ubiquitously expressed on all cells. Agonism of the GR can inhibit the activity of pro-

inflammatory transcription factors such as NF-κB and AP-1, which results in decreased antigen presentation, cytokine production and cell proliferation [17, 18].

Historically, corticosteroids and their synthetic derivatives have been (generally in tandem with other immunosuppressants) used as first-line maintenance therapy in transplant recipients. Furthermore, they are also often employed (in high doses) to treat active episodes of acute rejection. Long-term complications associated with corticosteroid therapy are numerous and can affect any portion of the body. Notable adverse effects include the increased risk of opportunistic infections, impaired wound healing, osteoporosis and iatrogenic Cushing's syndrome [17, 18].

1.2.2 Anti-Metabolites: Azathioprine and MMF

Azathioprine (AZA, also known as 6-mercaptopurine) was initially discovered to have antipurine activity in the bacteria *Lactobacillus casei*. AZA is noted as the first drug to allow for enhanced survival of transplant recipients (up to several weeks) in the 1960s and was later used in combination with glucocorticoids to allow for even longer allograft survival (several years). Though the exact mechanism of AZA is poorly understood, it is known to become incorporated into nucleic acids causing suppressing of purine synthesis, ultimately leading to inhibition of RNA and DNA synthesis and decreased cell proliferation. Because of the generic ability of AZA to suppress proliferation in all cells, it has many toxic side effects including bone marrow suppression [19, 20].

Mycophenolic acid (MMF) is yet another antiproliferative immunosuppression drug that is now generally used in most centers instead of azathioprine due to its more favorable toxicity profile and specificity for lymphocytes [21-24]. From a mechanistic standpoint, MMF is an inhibitor of inosine monophosphate dehydrogenase-2 (IMPDH2), which is the rate-limiting enzyme in *de novo*

guanine synthesis [24]. More specifically, IMPDH2 activity is increased in highly proliferating cells as well as in tissues with rapidly proliferating cell populations (such as T cells and B cells). As such MMF's inhibition of IMPDH2 allows for the decreased proliferation of T – and B-cells, without the collateral damage that is seen with AZA (namely suppressed bone marrow proliferation) [24]. Compared to AZA, complications with MMF are mild and include leukopenia, gastritis, esophagitis, and opportunistic CMV infection [21, 22].

1.2.3 Calcineurin Inhibitors - FK506 and Cyclosporine

Cyclosporine (CsA), implemented by R.Y. Calne in 1979, dramatically changed the landscape of organ transplantation and is responsible for transplantation becoming the gold standard treatment for end-stage organ failure [25, 26]. Mechanistically, CsA can suppress T cell activity via inhibition of the intracellular enzyme calcineurin. Under normal circumstances, calcineurin dephosphorylates a transcription factor known as the nuclear factor of activated T-cells (NFATc), which subsequently upregulates expression of IL-2 and other pro-inflammatory cytokines. By inhibiting the function of calcineurin, CsA can reduce expression of pro-inflammatory cytokines necessary for the activity of effector T cells. Though efficient in reducing the incidence of acute rejection, CsA has several severe side effects including end-organ toxicity, diabetes, hypertension and neurotoxicity [25, 26].

FK506 (also known as tacrolimus) was developed as a next generation calcineurin inhibitor in the advent of the success of CsA. Described in 1987 by Kino and colleagues, FK506 is a macrolide antibiotic produced by *Streptococcus tsukubaensis* [27]. From a mechanistic standpoint, it can bind to FK506 binding protein (FKFBP12). The resulting FKBP12-FK506 complex is then

able to inhibit calcineurin, resulting in decreased IL-2 and pro-inflammatory cytokine expression (analogous to CsA) [27-29]. Of importance, FK506 has been shown to be 10-200 times more potent than CsA, allowing for FK506 to be a superior alternative to CsA for preventing episodes of acute rejection, and even allowing for FK506 to be used as monotherapy in kidney transplantation [30, 31]. Similar to CsA, FK506 has numerous side effects including opportunistic infection, diabetes, nephrotoxicity and neurotoxicity [32-35].

1.2.4 Rapamycin

Rapamycin (also referred to as sirolimus) was initially discovered in the 1970s as a fermentation product of *Streptomyces hygroscopicus* in the Easter Islands with antifungal properties [36]. It was not until the discovery of the calcineurin inhibitor FK506 that researchers began to reevaluate rapamycin as a potential immunosuppressive agent [37]. Aside from having a structure similar to FK506, Rapamycin is also known to bind to the same intracellular target as FK506 (FKBP12) [38]. Upon binding FKBP12, the Rapa-FKBP12 complex is able to inhibit the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase. By inhibiting mTOR, rapamycin can regulate cell proliferation by preventing cell cycle progression from the G1 to the S phase and inhibit protein synthesis [38]. Furthermore, inhibition of mTOR renders T cells less responsive to multiple proliferative signals including IL-2 signaling [37, 38]. Unfortunately, these are not exclusive to cells of the immune system, and consequently, rapamycin can interfere with fibroblast function resulting in impaired wound healing [37, 38]. Other side effects observed with rapamycin include life-threatening pneumonitis, proteinuria and in some instances diabetes [37-41]. Despite these adverse effects, rapamycin has been shown to have negligible nephrotoxicity (a significant advantage over its calcineurin inhibitor counterparts). Because of this feature, rapamycin is often

used in patients with impaired renal function [37, 39]. In addition to its ability to affect T cell proliferation, rapamycin is also able to influence dendritic cell (DC) function, driving DCs toward a more tolerogenic phenotype. Furthermore, rapamycin has also been shown to synergize with cytokines such as TGF-β and IL-2 to induce for suppressive lymphocytes [38, 42-45].

1.2.5 Costimulation Blockage

T cell activation requires three well-described signals Figure 2. Signal one consists of TCR binding to the antigen in the context of MHC class II molecules on antigen presenting cells (APCs). Signal 2 (referred to as co-stimulation) consists of the binding of CD80 and CD85 on the surface of APCs to CD28 co-stimulatory molecule on T cells. Signals 1 and 2 lead to cytokine expression and lymphocyte proliferation, referred to as step 3 [46, 47].

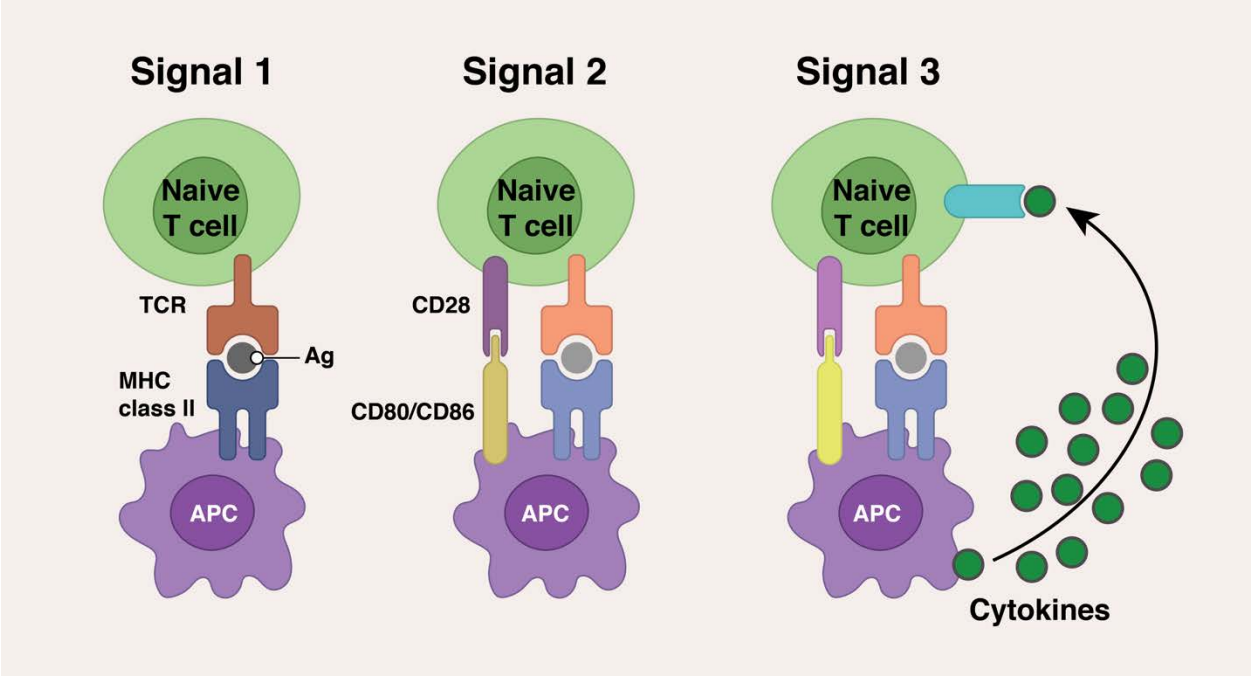

Figure 2. T cell activation requires three distinct signals.

Belatacept is a fusion protein consisting of the extracellular domain of cytotoxic T-lymphocyte antigen 4 (CTLA4, a protein receptor also found on T cells that binds CD80/86 and downregulates the immune system) linked to the FC fragment of human IgG1 [48]. Of note, belatacept is the first biologic agent approved by the FDA for maintenance immunosuppression in transplantation (renal) [46, 47, 49]. Belatacept acts as a selective co-stimulation blocker of T cells binding to both CD80 and CD86. Regarding affinity, belatacept can bind CD80 with a 5000-fold higher affinity to CD86 with a 2000-fold higher affinity when compared with CD28 [46, 47, 49]. When compared with CsA in trials, patients receiving belatacept had much better preservation of renal function, but also showed a higher incidence of acute rejection [48, 49]. Moreover, patients receiving belatacept also experienced better cardiovascular and metabolic endpoints when compared with those receiving CsA. Taken as a whole, belatacept treatment appears to obviate

some of the more deleterious side effects associated with calcineurin inhibitors and (despite the increased incidence of acute rejection) may result in better outcomes in the long term [48, 49]. Large studies with longer follow up will be necessary to elucidate this hypothesis.

1.3 INDUCTION THERAPY

The goal of induction therapy in transplant immunology is to initiate immunosuppression with potent and specific drugs [50, 51]. Administration of induction therapy occurs postoperatively or in some cases pre or perioperatively in anticipation of the transplant. The combined use of induction therapy with conventional maintenance therapy (usually, calcineurin inhibition) is imperative during the immediate postoperative period to prevent T cell activation from the early infiltration of donor cells into the recipient, initial allorecognition of the graft, and tissue injury from ischemia-reperfusion injury. Ultimately the use of these drugs reduces the incidence of acute rejection and decreases the initial dose of maintenance immunosuppression [50, 51].

1.3.1 Anti-Lymphocyte Globulin

Antilymphoctye Globulin (ALG) is a polyclonal antibody produced by immunizing rabbits or horses with human lymphocytes and was the first biologic agent used for induction therapy [50, 51]. Treatment with ALG results in the depletion of lymphocytes via a variety of mechanisms including opsonization, phagocytosis, lysis, and clearance via the reticuloendothelial system. After treatment with ALG, complete immune reconstitution has been reported to take several months [50, 51]. Because ALG is a heterogeneous non-human biologic agent, the formation of anti-

antibodies is a possible side effect, along with cytokine release syndrome, serum sickness, chills, and fevers. Due to advances in the development of more specific monoclonal antibodies (discussed below), the use of ALG is less ubiquitous [50, 51].

1.3.2 Basiliximab

Basiliximab is a humanized IgG mouse monoclonal antibody to the α chain (CD25) of the IL-2 receptor of T cells. Unlike ALG, basiliximab is not a T cell depleting agent, but rather it competitive inhibitor of the IL-2R. Antagonism of the IL-2R prevents T cell activation and proliferation while simultaneously bypassing the side effects associated with cell lysis and destruction [50, 51]. Because basiliximab is a chimeric (approximately 70% human, 30% murine) antibody, it is less immunogenic than nonhuman antibodies. Therefore neutralization is less of a concern. Basiliximab has an excellent safety profile, with clinical trials reporting the frequency of adverse reactions being comparable to placebo. Concerning its clinical efficaciousness, several studies have shown that basiliximab induction therapy, when compared to rabbit ALG, resulted in significantly lower rates of serum sickness, CMV infection, leukopenia, and thrombocytopenia [50, 51]. Mechanisms and cellular targets of the aforementioned immunosuppressive drugs mentioned in this chapter are illustrated in Figure 3.

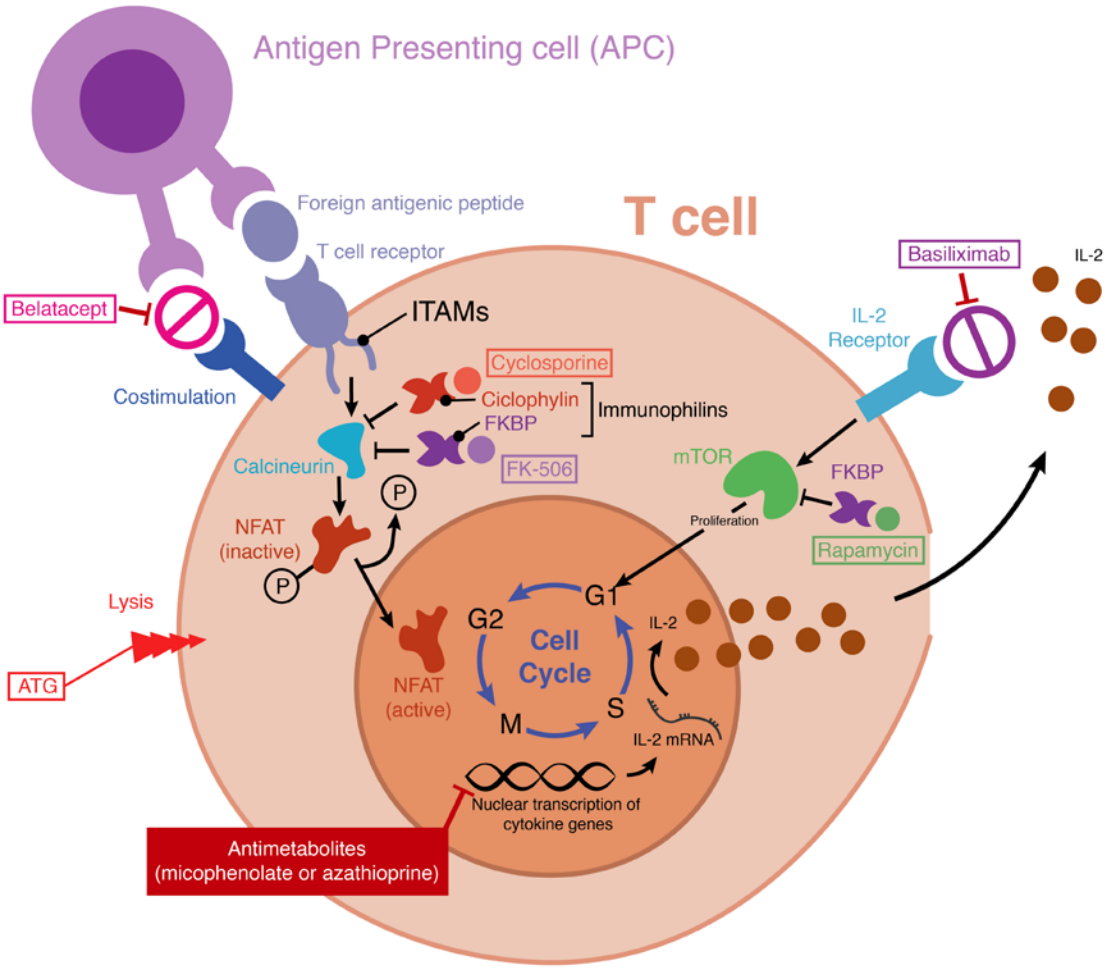

Figure 3. Mechanisms associated with T cell-mediated immunosuppression.

1.4 THE SHORTCOMINGS OF CURRENT GOLD STANDARD IMMUNOSUPPRESSIVE TREATMENTS

While the current lineup of immunosuppressive agents that are administered to transplant patients, allow for improved graft survival times, this is not without cost to the patient. As alluded to above, most forms of immunosuppression currently used are associated with significant toxicity and side effects including nephrotoxicity, opportunistic infections, diminished tumor immune-surveillance, serum sickness, anaphylaxis and neurotoxicity [33, 35, 40, 41, 50]. Additionally, the systemic delivery (either P.O. or IV) of these agents is often associated with unpredictable blood levels of the drug, leading to the presence of toxic (peak) or non-therapeutic (trough) concentrations. Taken together, organ transplantation is not technically a "curative" intervention, but rather patients are trading one chronic condition (end-stage organ failure) for another chronic condition (becoming a transplant recipient patient).

Another barrier to improved patient outcomes after transplantation is the issue of patient compliance and nonadherence. This should not be of surprised based on the laundry list of side effects listed above as well as the daunting dosing frequency of conventional immunosuppression protocols. While, there are multiple variables that can impact the degree of nonadherence (socioeconomic status, gender, education level), the incidence of nonadherence in some studies is as high as 68% [52]. Moreover, patient nonadherence is associated with poor outcomes, graft failure and increased costs to hospitals, patients, and payers [52-55].

The shortcomings described herein (namely toxicity and nonadherence), underscore the importance of improving current therapies and developing future technologies to improve patient and graft outcomes in the context of allotransplantation. Indeed, there are multiple emerging fields from multiple disciplines that will present unique solutions to the issues and shortcomings just

described. The proceeding sections of this chapter will describe some of these technologies, specifically cell therapy, and controlled drug delivery (with an emphasis on micro and nanoparticle-based controlled delivery). While fundamentally different these technologies present attractive treatments in the context of transplant immunology that are very similar at their core, specifically the minimization or elimination of immunosuppression and the promotion of allograft survival

1.5 REGULATORY T CELLS – MEDIATORS OF HOMEOSTASIS

The previous sections aimed to highlight the success and frustration of modern immunosuppression. While the field has advanced considerably since the days of whole-body irradiation and high dose corticosteroids (Figure 1), modern immunosuppression is still fraught with a well-established host of side effects. This is likely due to the fact that every clinically used immunosuppressive agent functions by blocking or suppressing a given pathway that is associated with lymphocyte proliferation [4, 6]. When compared the vast complexities and redundancies of the human immune system, such an approach (the blocking of a single or even multiple pathways) seems almost primitive. Unsurprisingly, our bodies (and specifically our immune systems) have evolved a whole host of mechanisms to regulate inflammation and promote homeostasis.

1.5.1 Treg History and Characterization

Indeed, over the past three decades, immunologists have made considerable strides in developing an understanding of the regulatory, or anti-inflammatory aspects of the immune system [56, 57]. Specifically, it was initially discovered that immune system contains suppressive mechanisms mediated by “certain T cells” that act to prevent self-reactivity [58-61]. While this seems logical with the knowledge, we have in the present day, at the time this suggestion contradicted dogma. Further studies demonstrated that suppressive T cells could be induced when presented with antigenic simulation in addition to certain cytokines such as TGFβ and IL-10, however despite their function, these cells did not express any markers that distinguished them from other T cells [62]. The lack of specific markers that could allow for isolation intensified skepticism that such suppressive T cells actually existed. Additional studies by Mason and Sakaguchi implicated CD45 and CD5 (respectively) as potential cell surface markers, however, these proved to only be species-specific [58, 60, 61]. Ultimately, it was not until 1995 that Sakaguchi and colleagues identified CD25 as a surface protein marker for these described suppressive T cells, which are now referred to as regulatory T cells (Treg) [63].

1.5.2 Treg Phenotype and Function

The identification of CD25 as a cell surface marker for Tregs allowed for extensive studies to be conducted on this newly identified cell type, and investigators were able to identify a panel of other markers that were also discerning for Treg [64, 65]. Of note is CTLA-4 (cytotoxic T lymphocyte-associated protein 4) which is a receptor found on Tregs that functions as an immune checkpoint and binds to co-stimulatory molecules found on the surface of antigen-presenting cells, effectively acting as an "off" switch. Tregs were also shown to express a host of chemokine receptors such as CCR4, CCR5, and CCR7 at various levels depending on their state of activation as well as their propensity to migrate to various sights *in vivo*. While the markers described thus far are characteristic of Tregs, they are all expressed by either naïve or effector T cells as well (albeit they are expressed at lower levels). At present, a single, specific cell surface marker for Tregs has not yet been described or identified.

While there exists a panel of cell surface markers that are overexpressed on Tregs, it is the intracellular transcription factor forkhead box P3 (FOXP3 also known as scurfin) that is the true master regulator of the regulatory pathway associated with Treg development and function [65]. This was first highlighted by three independent laboratories in 2003 which demonstrated evidence that FoxP3 was responsible for both Treg phenotype and function [66-68]. They further demonstrated that knockdown of FoxP3 resulted in failed Treg development and impaired suppressive function, and the induction of FoxP3 in naïve T cells resulted in enhanced suppressive function in the induced cells [64].

Currently, Tregs are most commonly identified as $CD4^{+}CD25^{hi}FoxP3^{+}$ lymphocytes. Furthermore, they can be subdivided into two distinct populations, specifically natural Tregs (nTreg) and induced Tregs (iTreg) [64, 65]. nTreg arise in the thymus and have been shown to

express T cell receptors specific for self-antigens (implicating a role for nTreg in autoimmunity). iTregs are induced *de novo* from naïve T cells in the periphery often in the prescience of TGFβ, IL2 and retinoic acid and antigenic stimulation. Further, it has also been shown that iTregs can be induced *in vivo* by nTregs in phenomena described as infectious tolerance [64, 65]. With respect to differentiating iTreg vs. nTreg, it has been demonstrated that nTreg exhibit higher expression of programmed cell death-1 (PD-1), neuropilin 1 (Nrp1) and Helios. However, it should be noted that individually, none of these markers possess the specificity to distinguish between nTreg and iTreg [69].

Functionally, Tregs exert their function through multiple pathways which can be broadly categorized as contact-dependent and contact-independent. For instance, Tregs mediate immune hyporesponsiveness in a contact-dependent fashion via expression of CTLA-4 which interacts with antigen-presenting dendritic cells (DCs) and induces these DCs to adopt a suppressive phenotype, rendering them unable to activate naïve T cells (and even promote apoptosis of pro-inflammatory T cells in some instances) [64, 65]. In addition, Tregs (depending on the nature of the type of inflammation) are also thought to secrete a number of different immunosuppressive cytokines and soluble factors including secretion of the suppressive cytokines such as IL-10 and TGF-β, which do not require direct cell contact. The secretion of these factors leads to a decrease in immune cell proliferation and local suppression of inflammatory cytokine production. Further, the presence of these mediators (especially TGF-β) has been known to induce both naïve and effector T cells to differentiate into a suppressive Treg phenotype [64, 65].

1.5.3 Treg Cell Therapy

Due to their unique and effective capability of regulating inflammation and promoting homeostasis, regulatory T cells are considered an extremely promising cellular-based immunotherapy for regulating inflammation in numerous pathologies including autoimmunity and transplantation [70-74]. To use Tregs as a cell-based therapy in the clinic, they must be isolated from the patient (typically from peripheral blood), expanded in *ex vivo* systems, and then be reinfused into the patient. Further, Good Manufacturing Product (GMP) facilities are necessary as well, to obviate safety concerns and ensure that a clinically relevant number of functional Tregs are generated [70, 71, 73]. Unsurprisingly, multiple groups are actively using Tregs to treat a number of conditions in humans including Crohn's Disease, Type 1 Diabetes, and Graft Versus Host Disease [75-78]. At present, there have been 620 trials of Treg cell therapies listed on https://clinicaltrials.gov/, of which 106 involve transplantation.

Though it could be argued that Tregs (as a cell therapy) have the potential to be far more effective than any single drug (in the context of immunotherapy), the prospect of using *ex vivo* expanded Tregs as a cell therapy is faced with a litany of complications and drawbacks. First, as mentioned above, this type of therapy would require the use of GMP facilities, and it would need to be demonstrated that Tregs being generated in said facilities are expressing FoxP3 and not expressing or secreting any proinflammatory cytokines [71]. Despite the number of trials using Tregs as cell therapy, there exists no consensus procedure for isolating $CD4^+CD25^{hi}FoxP3^+$ Tregs [79]. Indeed, to date there have been three separately defined categories of GMP grade clinical Treg: first generation ($CD4^+CD25^+$); second generation, bonafide Treg ($CD4^+CD25^+CD127^{low/-}$), and third generation naive Treg ($CD4^+CD25^+CD127^{low/-}CD45Ra^+$) [79]. Further, due to the scarcity of circulating Tregs (less than 2% of all Tregs are found in the periphery), any Tregs

isolated from a patient would need to be cultured and expanded *ex vivo* to generate a clinically relevant number of cells to reinfuse [80]. Finally, there are also a growing amount of studies addressing the propensity of Tregs to trans-differentiate into harmful effector T cells and the instability of FoxP3 as a transcription factor [81, 82]. Taken as a whole, Tregs represent a unique population of cells that have the possibility to be a transformative technology in the fields of immunotherapy and transplant immunology [64, 65, 70]. However, given their complexity, it is not surprising that methods to isolate and expand Tregs are also incredibly complex and may be cost prohibitive. Ultimately, the development of alternative methods that can harness the powerful immunoregulatory potential of Tregs, especially in the context of transplant immunology, are warranted.

2.0 DELIVERY OF IMMUNOSUPPRESSION USING MICRO AND NANOPARTICLES

The future success of the field of transplant immunology centers upon the resolution of many of the current shortcomings with conventional immunosuppression that have been described in the previous chapter. The utilization of technology developed in the field of controlled drug delivery has the potential to combat many of the shortcomings with current clinical immunosuppression (namely drug toxicity and patient compliance). At its most basic, controlled release technology can be defined as a collection of methods and tools for autonomously delivering an encapsulated drug at a given rate with precise timing, either locally or systemically. Indeed, this presents many possibilities for improving the effects of pharmacological therapies.

From a systemic standpoint, all pharmacological agents possess a "minimal effective concentration," below which there are no therapeutic effects as well as a "minimal toxic concentration" above which, a patient may be subject to undesirable toxic side effects. The range in between these two concentrations is often described as the "therapeutic window." To improve the therapeutic effects of a drug and minimize toxicity it is imperative to maintain drug concentration within the therapeutic window for the duration of treatment [83]. This is often problematic, especially when a drug possesses a particularly narrow therapeutic window. Drugs given in conventional forms (e.g., orally or IV) are distributed throughout the entire body, reaching the site of action often at an accelerated rate and exceeding the minimal toxic concentration. Because conventional drugs are not given as a continuous supply, and the human body has mechanisms to clear the drug (generally hepatically or renally), the concentration of these drugs then decreases below the minimal effective concentration. In many cases, the therapeutic range of

a given drug is only attained for limited time frames. Indeed, systemic controlled drug delivery aims to address issues associated with keeping a drug within its therapeutic window. This is often achieved by encapsulated or incorporating a drug within a polymer matrix, but can also be accomplished using coatings, reservoir systems, and osmotically controlled systems (to just name a few). In addition to systemic delivery, controlled release systems also possess the unique ability to provide local or regional delivery of a particular agent to target tissues (enhancing the effectiveness of the agent while concurrently keeping it away from nontarget tissues that could experience unwanted toxicity).

There are also numerous controlled release-based dosage forms based on these available including tablets, capsules, patches, films and micro- and nanoparticles (MNPs). While these formulations all possess varying levels of effectiveness, MNPs have considerable advantages over the other types of formulations, especially as it pertains to local, regional delivery. MNPs are easily injected via clinically used needles into target tissues or intravenously, can access tissues that are inaccessible for certain drugs (such as the Central Nervous System) and are easily adapted into other delivery mechanisms such as inhalation or topically [83, 84].

Multiple techniques exist for fabricating drug loaded MNPs such as spray drying, coacervation techniques, droplet microfluidics and solvent evaporation techniques. The latter is the primary fabrication technique employed in future chapters of this thesis and are described in Figures 3 and Figure 15.

The two most pertinent applications of MNPs will be highlighted in the preceding sections of this chapter are local delivery for local therapy and local delivery for systemic therapy [84]. When considering local therapy for local delivery, this application aims to reduce global toxicity and increase the effectiveness of a drug by delivering it directly to cells or tissues of interest. In

this particular application, it is possible to imagine scenarios in which larger MNPs are used and injected into a given tissue as a depot (maintaining high tissue levels of the drug but low systemic levels) or much smaller (less than 3μm) MNPs are used which can be phagocytosed to delivery drugs intracellularly. This application is particularly attractive when dealing with drugs that have a well-defined, localized tissue or cellular target and have a particularly unfavorable toxicity profile (e.g., chemotherapeutics and transplant rejection medications). Conversely, local delivery for systemic therapy applications has been designed in which MNPs are designed to release drugs into the systemic circulation (analogous to continuous intravenous infusion). This particular application is especially attractive in that it can reduce the frequency of administrations (which could obviate issues associated with patient compliance) while still keeping the systemic level of the drug in the therapeutic window [84].

Given the versatility of MNP drug delivery systems, there has been much interest in improving current immunotherapies (described in Chapter 1) by incorporating them into MNP delivery systems. Though there are numerous studies detailing MNPs in cancer therapy, vaccine development and infectious disease [84-86], the remaining sections of this chapter will focus on small molecule eluting MNPs and their applications in transplant immunology.

2.1 CALCINEURIN INHIBITOR MNP RELEASE SYSTEMS

As mentioned in Chapter 1, CNIs currently remain the mainstay of most clinically employed immunosuppression protocols. Due to toxic side effects associated with systemic CNI therapy and poor oral bioavailability, there has been significant interest in developing CNI based MNP delivery systems [26, 33].

Sanchez *et al.* were among the first to develop, characterize and test CsA MNP formulations [87]. In these studies, three groups of poly(lactic-co-glycolic acid) (PLGA), MNPs (30 μm, 1 μm, and 0.2 μm) were fabricated. *In vivo* murine studies demonstrated that after subcutaneous injection, the 1 μm and 0.2 μm MNPs displayed a significant burst phenomenon (i.e., high initial blood levels of CSA) whereas the 30 μm formulations lacked such a large burst, providing steady blood levels of drug for over three weeks [87-89]. Building on this study, Urata *et al.* developed control release CsA poly(L-lactic acid) (PLA) microparticles containing fatty esters capable of releasing CsA in vivo for up to 4 weeks (without an initial burst). These formulations were effective at reducing symptoms of arthritis when administered in a rat model [90].

Targeted delivery of CsA MNPs (to both cells and lymphatics) offers all of the advantages of having a depot release of CsA while simultaneously providing a higher local concentration of drug to the tissues and cells of interest. Yoshikawa *et al.* were able to design PLGA nanoparticles (average diameter 260 nm) that released CsA for 30 days [91]. Moreover, intramuscular injection of CsA nanoparticles (femoral region) yielded undetectable plasma levels of CsA, but concentrations in the femoral lymph nodes were nearly 20 times that obtained when administering soluble systemic CsA [91].

Previous studies have revealed that polyethylene glycol (PEG) coated PLA particles (when compared to PLA particles) administered orally delivered significantly higher amounts of radioactive antigen to the lymphatics [92]. Building on this finding, Azzi *et al.* described a method for conjugating CsA to PLA/PEG nanoparticles (average size 84 nm). These CsA/PLA/PEG NPs were able to suppress T cell proliferation in mixed lymphocyte reaction (MLRs) in a comparable manner to soluble CsA. Moreover, they were able to show that DCs could effectively phagocytose

and traffic NPs to the popliteal lymph nodes in a mouse model. Finally, NP-treated DCs were also able to suppress T cell proliferation (MLR) when compared to untreated DCs [93].

In addition to synthetic polymer-based MNPs, several groups have developed natural (biological material) based MNPs to release CsA. Natural materials can have advantages over their synthetic counterparts, including improved cytotoxicity, genotoxicity, and hemocompatibility. Woo *et al.* described hyaluronic acid microparticles that exhibited superior dissolution, solubility, and bioavailability characteristics when compared with soluble CsA [94]. Additional studies using chitosan and fibrin-based nanoconstructs have been described, however, applications to clinically relevant pathologies have not yet been explored [95, 96].

Controlled release formulations of FK506 have also been explored due to FK506's potency and superiority to CsA [30, 31]. Sustained delivery of FK506 from PLGA-based MNPs is reported to be effective at preventing liver and islet allograft rejection and mitigating the effects of colitis in rodent models [97-100]. Investigators have also developed FK506 releasing Poly-D,L-lactic acid (PDLLA) releasing microparticles that (when administered orally) home to the Peyer's patches and prolong small bowel transplant survival in a porcine model [101]. Significant research has also been conducted in the development of pH-sensitive FK506 MNPs, which can deliver FK506 to the colon [102, 103]. These delivery systems have been used to treat murine models of inflammatory bowel disease (IBD); however, such technologies could be used in the context of small bowel transplantation [99, 100, 104].

Very recently Gajanayake *et al.* tested the ability of a triglycerol monostearate (TGMS) enzyme-responsive hydrogel loaded with FK506 to prevent hindlimb allograft transplant rejection in a rat model[105]. Specifically, a one-time injection of FK506 loaded TGFMS was shown to prolong hindlimb survival across a full MHC barrier for > 100 days. Furthermore, it was

demonstrated that tissue levels of FK506 in treated animals were increased 10 fold over systemic levels [105]. While this study did not specifically utilize MNPs, it is possible to envision future applications of such a gel used in tandem (or as a carrier) with MNPs to provide dual delivery of immunosuppression.

2.2 RAPAMYCIN MNP RELEASE SYSTEMS

Rapamycin based MNP systems are an especially attractive therapy as Rapa has been shown to not only inhibit T cell proliferation but also inhibit DC maturation and function and enrich for suppressive FoxP3+ Regulatory T cells [37, 42, 43, 45, 106]. As such, technologies taking advantage of both of these characteristics have been described in the literature. Forrest *et al.* described injectable rapamycin-loaded poly(ethylene glycol)-b-poly(epsilon-caprolactone) (PEG-PCL) micelle constructs that could provide over 6 days of *in vitro* release [107]. Due to the described effects that rapa can have on DCs, several studies have focused on targeted delivery of PLGA based MNPs to DCs [108]. Jhunjhunwala *et al*. described rapa microparticles (3-4 μm) that are phagocytosed by DCs and release their payload intracellularly for up to three weeks. DCs treated with these rapa microparticles had a decreased ability to induce T cell proliferation, and showed down-regulation of surface co-stimulatory molecules [108]. Two other studies examined the delivery of rapa to DCs (both human and mouse) with smaller PLGA nanoparticles (150-450 nm). These studies demonstrated that rapa nanoparticles are effectively taken up by DCs and decreased expression of all maturation markers (Figure 2B). Furthermore, when compared with free rapamycin, rapa nanoparticle-treated DCs showed decreased immunostimulatory capacity [109, 110].

In addition to PLGA based MNPs, there have been reports of alternative materials used in the development of rapamycin drug delivery systems. Kauffman and associates recently described rapamycin-loaded, acetylated dextran microparticles that have several advantages over PLGA microparticles such as more tunable release kinetics and differential release in acidic vs. neutral conditions [111]. Furthermore, Yuan *et al.* were able to develop rapamycin load chitosan/PLA nanoparticles to prevent rejection in a rabbit model of corneal transplantation. Chitosan/PLA rapa nanoparticles were able to show superior precorneal retention (as compared to free rapa) and were able to delay corneal allograft rejection [112].

2.3 ANTIMETABOLITES MNP RELEASE SYSTEMS

In addition to intracellular delivery of rapamycin, there has been considerable interest in developing MNP based systems that can release the antimetabolite MMF. Shirali et al. described an MMF PLGA based nanoparticle system that appears to prolong full thickness murine skin transplants across a full MHC mismatch (BALB/c → C57BL/6), when compared with daily systemic administration of MMF, via an apparent PD-L1 mediated mechanism. Notably, this therapeutic result was achieved despite MMF nanoparticles containing 1000 fold less MMF than is typically required with systemic administration of MMF. Moreover, treatment with MMF nanoparticles resulted in no detectable toxicity, whereas recipients receiving conventional MMF developed iatrogenic cytopenias [113]. In a similar study, MMF loaded in nanoparticles containing cyclodextrins was shown to be capable of prolonging survival in a mouse model of lupus, when compared to free drug. The authors concluded that the enhanced ability of MMF nanoparticles to suppress inflammation could be attributed to nanoparticle uptake by DCs (Figure

2B) [114]. In yet another study from this group, researchers directly compared DC internalization ability of MMF PLGA nanoparticles to MMF nanoparticles [115]. The authors were able to show that while both MMF formulations were able to reduce production of proinflammatory cytokines and stimulatory surface markers, the MMF nanoparticles were more effective, likely due to their enhanced uptake by DCs. As a caveat, while the MMF nanoparticles were more efficiently taken up by DCs, similar immunosuppressive effects could probably be seen with MMF PLGA nanoparticles at the expense of using a higher dose of nanoparticles [115].

2.4 MNP DELIVERY OF GENETIC MATERIAL FOR IMMUNOMODULATION

In addition to the delivery of commonly used immunosuppressive agents, investigators have also explored the possibility of using genetic material to interfere with expression of target genes. It is known, double-stranded small interfering RNA (siRNA) or single-stranded antisense oligonucleotides, can silence or down-regulate expression of specific genes of interest in the inflammatory pathway. Due to the ubiquity of serum nucleases, direct infusion of these factors is an inefficient delivery method [116, 117].

MNP systems represent effective carriers for genetic material as they can protect the payload and in some instances delivery the given material intracellularly allowing for efficient gene silencing. While gene therapy and delivery using MNPs have been described extensively in the literature for the treatment of a multitude of diseases, there are few studies examining the use of MNP-based gene delivery to suppress inflammation. Giannoukakis and Trucco have used DC's treated with anti-sense CD40, CD80, and CD86 oligonucleotides for treatment of type 1 diabetes; this cell therapy approach is currently in clinical in trials [118]. Despite the success of this

approach, Giannoukakis and Trucco have also described a microparticle-based system for intracellular delivery of these same factors to DCs. Polyethylene glycol (PEG)/polyvinyl pyrrolidone (PVP) based microparticles loaded with the antisense oligonucleotides were prepared and injected subcutaneously to a site anatomically proximal to the pancreatic lymph nodes in nonobese diabetic mice [119]. These microparticles accumulated in both the pancreas and spleen after injection and could reverse type 1 diabetes in treated mice. Moreover, Tregs isolated from microparticle treated mice were able to prevent beta cell allorecognition when adoptively transferred to secondary recipients [119]. Given these convincing results, it is likely that this approach could be met with success if applied to organ transplantation as well.

3.0 BIOMIMETIC DRUG DELIVERY

Biomimetic bioengineering or biomimetics is a multidisciplinary field in which concepts from engineering, medicine, biology, and chemistry are applied to generate a synthetic material or system that mimics a similar material or system that is found in nature. This approach is rooted in the fact that living organisms represent the result of millions of years of evolution via natural selection. As such, it should not be surprising that nature has "engineered" solutions for countless problems for organisms such as exposure to extreme environmental conditions and self-assembly [120]. While it may seem that biomimetics is an emerging field, the concept of mimicking concepts seen in nature has existed for hundreds of years. While unsuccessful, the first descriptions of biomimetics can be attributed to drawings of a "flying machine" proposed by Leonardo da Vinci that were based on his observations of the anatomy of birds. Modern examples of biomimetics include velcro (drawing inspiration from burs), and neural network computing systems (mimicking the action of neurons) [120].

Biomimetics has also given rise to new technologies and materials that have drawn inspiration at the cellular and molecular level. Indeed, the cells of our bodies are continuously interacting with one another (and their environments) often exchanging enormous amounts information resulting in sophisticated responses from target cells. Hallmark examples of this type of communication include the interactions between naive T cells and antigen presenting cells resulting in the propagation (or regulation) of an immune response or the interactions between lymphocytes, chemokines secreted from distant cells, and endothelial cells resulting in lymphocyte extravasation towards a target sight in surrounding tissue. Central two these examples of cell-based information exchange are the delivery of biological factors with specific spatiotemporal context.

It should be noted that the context within which these factors is presented to a target cell is just as important as the actual composition of the delivered factors themselves. This is analogous to humans communicating with one another; it is not only the words that make up a sentence that are important but also the order in which they appear [121].

Biomimetic delivery systems using micro-and nanoparticles (MNP) can be designed to replicate the cellular interactions described previously. Due to their versatility, MNPs can be designed with a size and shape to mimic the way in which a cell presents biomolecules in vivo. Indeed, MNP delivery systems have been vigorously explored since Langer and Folkman's seminal work in 1978 demonstrating the sustained release of biochemically active macromolecules from polymers [122].

While MNP systems are effective at producing and sustaining therapeutically relevant levels of pharmacological agents (see chapter 2), MNPs can also be used to mimic paracrine signaling via the local presentation of growth factors and cytokines to act on target cells and eliciting a desired response such as proliferation or differentiation. Moreover, MNPs address limitations associated with the administration of a systemic bolus of paracrine signaling factors, namely the short half-life of proteins (minutes to hours) and the lack of local delivery. Indeed, by producing a sustained release, MNP systems can extend the therapeutic activity of biomolecules from minutes to days or even weeks, delivered to a target tissue. Numerous groups have utilized biomimetic MNP systems to mimic local, paracrine delivery of growth factors in cytokines. Notable examples include the delivery of IL-10 to suppress inflammation associated with inflammatory bowel disease as well as delivery of BMPs and VEGF to promote osteogenesis and angiogenesis respectively [123-127]. In addition to delivering individual factors, MNP systems that demonstrate higher levels of sophistication, have also been described which can

simultaneously delivery multiple factors, mimicking the actions of dendritic cells and certain tumors (which secrete multiple factors, to influence cells) [128, 129].

As well as paracrine delivery of individual and multiple factors, MNP systems can also be utilized to orchestrate cell trafficking via the establishment of physiological chemokine (cell recruiting protein) gradients. It is known that the movement of many cell types is directed by the prescience of various chemokines that are secreted by source cells which diffuse outward and establish a gradient, recruiting cells that express the cognate receptor for that specific chemokine. Cells of the immune system rely heavily on cell mobilization via chemotaxis to lymphoid tissue for antigen priming and stimulation. Furthermore, tumor cells have been shown to recruit suppressive cells as a means of immune evasion. Indeed, multiple groups have used MNPs to mimic this phenomenon [130, 131].

While this section aimed to serve as a primer of the emerging field of biomimetic drug delivery, the subsequent sections of this chapter will focus on the use of MNP based biomimetic drug delivery as a means to regulate and influence the immune system.

3.1 MNP SYSTEMS FOR ENRICHING REGULATORY T CELLS

It is well understood that the phenotypic fate of naïve T cells is determined by both soluble paracrine signals as well as T cell/APC contact in the immune synapse. Due to the potent immunoregulatory characteristics of Treg, strategies to induce naïve T cells to adopt a Treg phenotype have the potential to promote self-tolerance in the context of autoimmunity, or acquired tolerance in the context of allotransplantation [132-135]. MNP based systems that deliver cytokines and pro-regulatory factors to promote Treg induction and expansion have been described

by several groups (Figure 4D). For example, investigators have developed targeted anti-CD4 coated PLGA based nanoparticles that release leukemia inhibitory factor (LIF), an IL-6 like cytokine shown to induce FoxP3 expression in T cells [134, 136, 137]. These CD4 targeting LIF nanoparticles were capable of expanding FoxP3+ cells *in vivo* in treated mice and expanding nonhuman primate Tregs *in vitro* [136]. Additionally, these same nanoparticles prolonged vascularized heart allograft survival across a full MHC mismatch when compared with controls receiving no treatment [136]. In yet another study, investigators were able to prevent β-islets cell transplant rejection across a full MHC barrier and promote normoglycemia in diabetic mice by tethering LIF eluting PLGA nanoparticles to β-islets [138]. Jhunjhunwala et al. describe another mimetic MNP based system that can induce/expand Tregs. In this study, they demonstrated that the combination of TGFβ, IL-2, and rapamycin, (both soluble and in sustained release PLGA microparticle formulations) could convert both mouse and human naïve CD4+ T cells to FoxP3+ Tregs *in vitro* [128]. Specifically, after 4 days of co-culture with naïve T cells, this triple cocktail microparticle therapy efficiently induced FoxP3 expression in 80% of T cells. Importantly these microparticle-induced Tregs were capable of robust proliferation and expressed key canonical Treg cell surface markers (CD25, FR4, and GITR) [128]. Based on the results to date, it is possible that such Treg expanding and inducing microparticle formulations could serve as an off the shelf therapy for suppressing aberrant inflammation in the context of transplant immunology and autoimmunity.

While the idea of regulatory cell expansion and induction is an effective strategy to promote homeostasis, recruiting endogenous regulatory cells with particle-based “homing” systems is another viable strategy to enrich cells in a given location [106]. Indeed this concept is often exploited in nature by cells that secrete chemokines (chemoattractant cytokines) to recruit

cells expressing the corresponding chemokine receptors to the source of the gradient. As a hallmark example, it has been shown that a variety of tumor cells secrete the chemokine CCL22, which appears to effectively recruit Tregs, which express the associated CCR4 receptor [139]. This local recruitment of Treg to the site of the malignancy is thought to enable tumor-specific immune evasion. Based on such phenomena, it is possible to envision strategies to recruit Tregs using CCL22 and induce therapeutically-relevant, local immunological hyporesponsiveness [140]. For instance, pancreatic islet cells have been transfected with an adenovirus designed to overexpress CCL22, as a means to recruit Tregs to the pancreas and prevent autoimmune diabetes in a mouse model [141].

Building on these findings, controlled release of CCL22 from PLGA microparticles was shown to promote site-specific recruitment of endogenous Tregs *in vivo* (Figure 4D) [131]. Specifically, CCL22 microparticles were able to recruit adoptively transferred Tregs in a mouse model to the site of microparticle injection and concurrently delay rejection of transplanted allogenic cells (which were also injected at the site of microparticle administration) [131]. Moreover, Treg homing to a locally placed depot of CCL22 microparticles has been shown to obviate the inflammatory effects of periodontal disease in both murine and canine models [142].

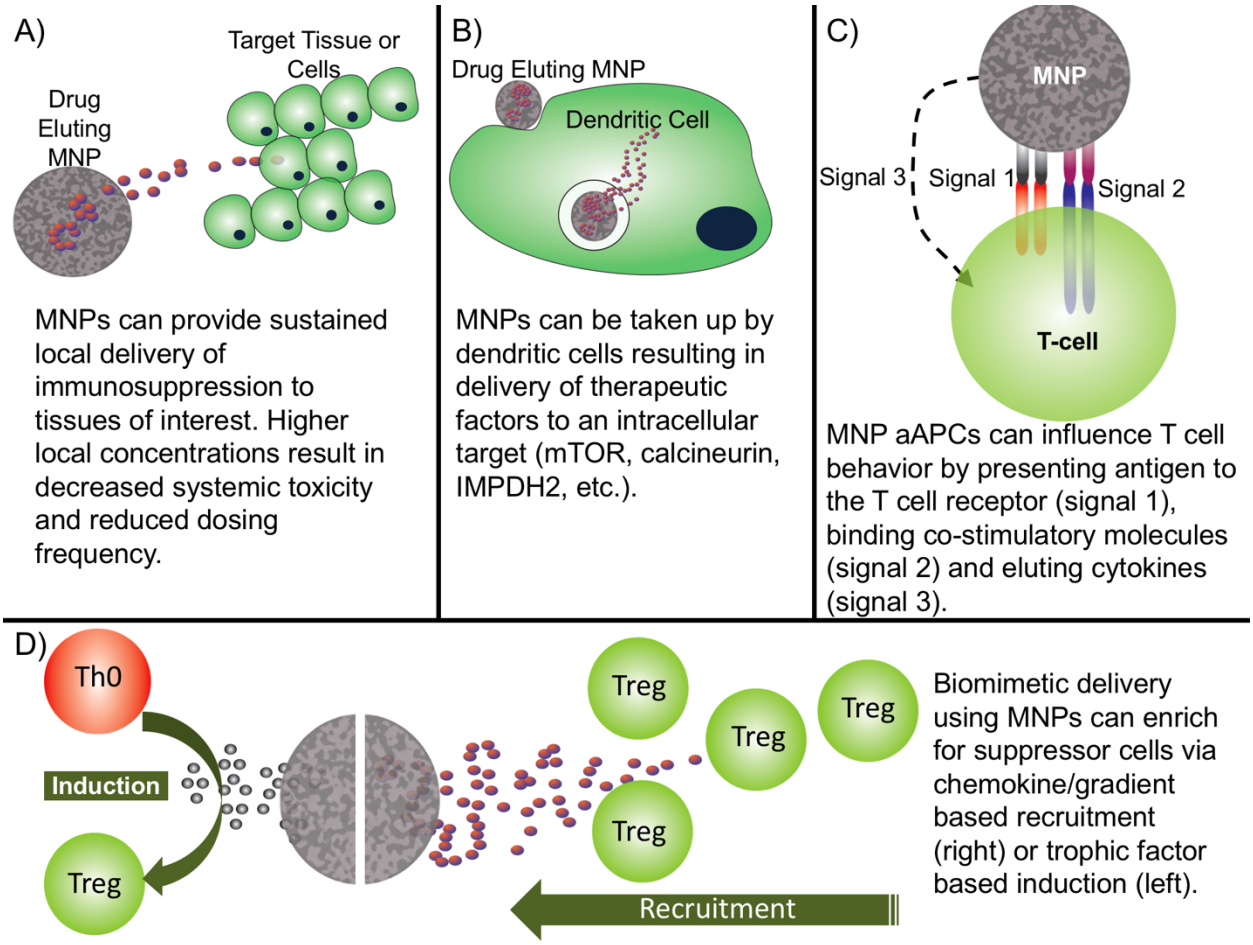

Figure 4. Delivery routes and possible mechanisms associated with micro and nanoparticle (MNP) mediated drug delivery. At their most basic application, MNPs can present target tissues and individual cells with a local, sustained and higher concentration of drugs then possible with systemic delivery (A-B). More complex and biomimetic applications of MNPs have also been described in which they can influence lymphocytes through a number of mechanisms (C-D). Adapted from Fisher, J.D. et. al., 2015 [14].

3.2 MNP SYSTEMS TO INDUCE TOLEROGENIC DENDRITIC CELLS

Building on the concept of multifactor microparticle-based delivery, Lewis et al. recently demonstrated that combined (intracellular and extracellular) delivery of cytokines and small molecules to DCs could prevent DC maturation, thereby inducing a more tolerogenic phenotype [129]. Two classes of PLGA microparticles were designed; phagocytosable microparticles loaded with either rapamycin or retinoic acid (RA) and unphagocytosable microparticles loaded with either TGFβ or IL-10. Four distinct combinations of microparticles (Rapa/IL-10, Rapa/TGFβ, RA/IL-10 and RA/TGFβ) were tested, when cultured with DCS they were shown to decrease levels of surface expression of MHC II, CD80, and CD86 and were resistant to LPS stimulation. Furthermore, DCs treated with these dual microparticle systems were able to suppression T cell stimulation in mixed lymphocyte reactions and skew T cells toward a regulatory phenotype. Importantly, in all experiments, it was shown that combinatorial parings of immunosuppressive microparticles were more effective than microparticles releasing any of the respective single factors [129].

3.3 ARTIFICIAL ANTIGEN PRESENTING CELLS

It is known that DCs play a key role in the initial steps of T cell activation, namely via antigen presentation (signal 1) in the context of signal 2 (costimulation) and 3 (paracrine cytokine signaling). Accordingly, there is great interest in developing synthetic particle-based materials to mimic the function of DCs in this process, allowing for specific T cell targeting for drug delivery (Figure 4C) [143, 144]. Referred to as “artificial antigen presenting cells” (aAPCs), these MNP

particles seek to engage the TCR receptor in a similar manner as a natural APC and elicit a given response (Figure 2C) [145]. In general, most aAPC constructs have a high density of surface-bound anti-CD3 and anti-CD28 ligands for the T cell receptor (CD3) and the costimulatory receptors (CD28) (signals 1 and 2). Further, there are descriptions of aAPCs that (in addition to the aforementioned surface coatings) are also loaded with cytokine (signal 3). Though anti-CD3 can serve as a nonspecific activator for all T cell receptors, there are also descriptions of aAPCs that can present a given peptide in the context of an MHC class II complex (analogous to what is observed in nature), thus providing an antigen-specific response [146].

The ideal aAPC should possess three characteristics, namely, it should be 1.) Easily modifiable to allow for a variety of surface modifications, 2.) Capable of controlled release of cytokines when engaged with a T cell and 3.) Available in an off the shelf formulation [145, 147]. Steenblock et al. were the first to describe such an all-inclusive aAPC [145]. Using PLGA as the base polymer, these aAPCs consisted of surface-bound anti-CD3, and anti-CD28 as well as encapsulated soluble IL-2 (signals 1, 2, and 3,). Surface modification was carried out by incorporating an avidin-palmitic acid conjugate into the PLGA emulsion (thus allowing the palmitate to interact with the hydrophobic PLGA core and the avidin residing on the surface) allowing for biotinylated anti-CD3/CD28 or peptide/MHC complex conjugation to the particle surface. This combination of antigen presentation, costimulation and paracrine delivery of IL-2 was able to enhance *in vitro* CD8+ T cell proliferation by a factor of 45 when compared with addition of exogenous IL-2 [145]. Building on this observation, further studies showed that the spatial and temporal characteristics of paracrine IL-2 release from an aAPC could have differential effects on CD8+ and CD4+ cells. Specifically, delivery of IL-2 upon aAPC contact with the TCR complex promotes IL-2 accumulation in the APC/T cell immune synapse, resulting in robust CD8+

proliferation, but activation-induced apoptosis in CD4+ populations [147]. Interestingly, this result could not be replicated even with the addition of 1000 fold exogenous IL-2, suggesting that a slow, sustained release along with the synaptic accumulation of IL-2 are responsible for this result [147].

Though the vast majority the applications of aAPCs in the literature focus on immunostimulation and effector T cell proliferation, it is easy to envision strategies that employ the use of aAPCs for immunoregulatory applications. Indeed, Francisco *et al.* were able to show that surface-bound PD-L1 on Dynabeads along with soluble TGFβ could enhance Treg induction when co-cultured with naïve T cells [148]. Schultz et al. were the first to describe "killer aAPCs" that could target and delete antigen-specific T cells [149, 150]. These killer aAPCs consisted of microparticles with the apoptosis-inducing ligands (anti-Fas ligand (anti-FasL)) and HLA-A_2 affixed to the particle surface [150]. When co-cultured with T cell populations, these aAPCs were able to promote antigen-specific T cell depletion in a FasL-dependent mechanism [150]. In another study, "killer aAPCs" were shown to prolong skin allograft survival (in a mouse model) via the deletion of antigen-specific, alloreactive T cells [151]. Given these promising results, it will be interesting to see how these therapies translate to solid organ transplantation.

The interactions between DCs and T cells in the immune synapse is a complex process, and the results from the aforementioned studies (while promising) underscore this complexity and the necessity of continued understanding of these interactions. Indeed, paracrine factor accumulation between cells and nanoparticles as well as the thermodynamic interactions between MNPs and cells has been described in the literature [152, 153]. Studies of this nature will continue to provide the necessary clues to developing an effective artificial cell construct. Given this complexity, the development of effective aAPCs for the next generation immunotherapeutics will rely on the continued collaboration between engineers and immunologists.

Table 1. Summary of reported MNP drug delivery systems for immunosuppression and regulation in the context of transplant immunology.

	Device Description	Key Findings	Ref.
Small Molecule MNPs	FK506 Eluting PLGA MPs	Subcutaneous injection of FK MPs could maintain therapeutic blood levels of drug and prolong murine liver and islet allograft survival.	97,98
	FK506 Eluting PDLLA MPs	FK506 MPs accumulate in Peyer's Patches after oral administration and prolong swine small bowl transplant survival.	101
	FK506 Loaded TGMS Hydrogel	A single injection of enzyme responsive FK506 TGMS hydrogel prolongs rat hind-limb transplant survival when compared to systemic FK506.	105
	Rapa Loaded PLA/Chitosan NPs	PLA/Chitosan NPs release rapa for over 8 days. NPs demonstrated excellent retention in procorneal area and prolong corneal allografts (rabbit) when compared to rapa eye drops.	112
	Rapa Eluting PLGA MNPs	Rapa MNPs can be taken up by DCs for intracellular rapa delivery. Treated DCs showed decreased immunostimulatory capacity and decreased expression of maturation markers.	107-109
	MMF Loaded PLGA NPs	Intraperitoneal injections of MMF NPs prolong murine skin allografts (across a full MHC barrier) and show a decreased toxicity profile when compared to systemic soluble MMF	113
	Antisense Oligonucleotide PEG/PVP MPs	Subcutaneous injection of MPs loaded with antisense CD40, CD80 and CD86 oligonucleotides reverse type 1 diabetes in NOD mice.	118
Biomimetic MNPs	LIF Eluting Anti-CD4 Coated PLGA NPs	LIF NPs coated with anti-CD4 target naïve T cells, expand nonhuman primate Tregs (*in vitro)*, expand murine Tregs *in vivo* and prolong murine heart allograft survival.	136
	CCL22 Releasing PLGA MPs	CCL22 MPs set up a chemokine gradient to recruit adoptively Tregs *in vivo* and can prevent inflammatory effects of periodontal disease in both murine and canine models.	131, 142
	Rapa, IL-2, and TGFβ PLGA MPs	The combination of RapaMP/IL-2MP/TGFβ MP is able to induce naïve T cells (mouse and human) toward FoxP3+ Treg phenotype *in vitro*.	128
aAPC MNPs	Anti-CD3 and Anti-CD28 Affixed to IL-2 PLGA MP	IL-2 MPs with anti-CD3 and anti-CD28 coupled to the MP surface interact with CD8+ T cells *in vitro* and can induce robust proliferation.	145, 147
	Anti-Fas and MHC Antigen Affixed to Polystyrene MPs	Killer aAPCs coated with apoptosis inducing anti-Fas can selectively delete antigen specific T cells and prolong skin allograft survival in a mouse model.	149, 150

4.0 TRANSLATIONAL CONSIDERATIONS

Though the previously-described technologies are only in the *in vitro* development stages or *in vivo* preclinical studies, it should be noted that there are numerous MNP-based drug delivery systems, for other applications, that are both FDA approved and in clinical trials [154]. These systems utilize many of the materials such as PLGA, PLA and PEG and lipid-based liposomes. Nonetheless, these systems all consist of an existing FDA-approved drug packed into a drug delivery system [155]. The advantages of controlled and sustained release have been described (namely decreased toxicity and improved patient adherence), however translation of nonconventional drug delivery systems (including MNP loaded with genetic material, biomimetic protein delivery, and aAPCS) will likely be more cumbersome as these technologies would be treated as a new drug [156]. For example, translation of a drug delivery system based on an existing FDA approved agent can cost between 20-50 million dollars and take 3-4 years [156]. In contrast, development of a new drug can cost upwards of 500 million dollars over ten years [156]. Accordingly, it seems that in the immediate future, development of MNP systems that release FDA-approved immunosuppressive agents may have the easiest path to translation. This is not to say that the more complex delivery systems described are impractical, but a greater understanding of their toxicity profiles and clinical efficaciousness will need to be demonstrated before moving onto clinical trials.

While MNP systems that release FDA-approved immunosuppressives have an easier path to FDA approval, they also retain a host of issues that remain unresolved. The MNP systems currently approved by the FDA range have a range of indications including delivery of chemotherapeutics, antipsychotics, and antibiotics [155-157]. However, there currently exists no MNP delivery system for immunosuppressive agents (for any application). The successful translation of such systems will hinge on demonstration of the safety of sustained release of highly toxic drugs such as FK506. Furthermore, delivery of immunosuppressive drugs using MNPs could present an issue if patient exposure to the delivered drug was to be discontinued (for example a transplant patient presenting with an active opportunistic infection). A potential solution to this problem would be to delivery MNPs in an injectable solution that could solidify after administration (such as a thermally responsive or shear responsive hydrogel)[158, 159]. The MNP gel complex would remain intact, provide sustained release of a given material and be easily removed in the event that therapy would need to be discontinued.

The development of strategies for suppressing and modulating the immune responses following allotransplantation has improved considerably over the past 50 years. Rudimentary therapeutics such as corticosteroids and azathioprine that have global effects throughout the body have been replaced with more drugs that have T cell-specific targets, with fewer (although still considerable) side effects. The development of both controlled, targeted delivery of these drugs as well as fabrication of biomimetic systems to modulate the immune system have the ability usher in a new era in transplantation in which allotransplantation may represent a true cure for end-stage organ failure. In the proceeding chapters of this thesis, two approaches are put forth that utilize biomimetic controlled release microparticle systems that aim to locally enrich for suppressive Regulatory T cells (Treg) as a means to promote transplant tolerance.

5.0 REGULATORY T CELL RECRUITING MICROPARTICLES PROMOTE DONOR ANTIGEN SPECIFIC TOLERANCE IN VASCULARIZED COMPOSITE ALLOTRANSPLANTATION

5.1 INTRODUCTION

Vascularized Composite Allotransplantation (VCA), encompassing the transplantation of multiple tissue types derived from multiple embryonic origins is an emerging field of allotransplantation with more than 100 types of VCAs having been performed in the past decade (primarily hand and face transplants) [1, 160]. Still, VCA is hampered by the same issues faced by solid organ transplantation (SOT), chiefly allorecognition of donor tissue [161-163]. To combat this, patients are placed on systemic maintenance immunosuppression protocols consisting of at least 2-3 drugs that have a well-established deleterious sequelae (see Chapter 1.2). As such, there is a significant need in clinical VCA to explore alternative strategies for achieving graft-specific immune hypo-responsiveness while concurrently obviating concerns associated with lifelong immunosuppressive drug treatments (and their associated toxicity) [164].

One such strategy that has been increasingly explored is using the powerful immunoregulatory properties of regulatory T cells (Treg, Chapter 1.5). Indeed, there are multiple active clinical trials that utilize Treg cell therapy, however, the use of Treg in cell therapy is incredibly complex and fraught with a number of logistical and regulatory hurdles that were

described in Chapter 1.5.3. As an alternative, we have considered utilizing known mechanisms by which endogenous cells are able to locally enrich active, suppressive Tregs *in situ* by establishing a chemokine gradient to allow for directional migration of Tregs to a point site. Specifically, it is known that certain tumors are able to recruit circulating Tregs to the tumor milieu by releasing (secreting) C-C motif chemokine ligand 22 (CCL22), leading to Treg to the site of the tumor [165].

Using this immune-evasion strategy as inspiration, a safe, synthetic controlled release system capable of generating a sustained gradient of Treg-recruiting chemokine (CCL-22) from the site of placement *in vivo* was developed. This formulation, (referred to as Recruitment-MP), has been shown to recruit Tregs both *in vitro* and *in vivo*[131]. Furthermore, it has also been shown that Recruitment-MP can resolve destructive inflammation and significantly alleviate symptoms in animal models of both periodontal and dry eye disease [142, 166]. As a next step, Recruitment-MP was tested in a rodent hind-limb VCA model (a notoriously stringent model of inflammation). Specifically, it was hypothesized that Recruitment-MP could take advantage of the bodies' own sophisticated mechanisms of immune regulation in the context of allotransplantation as a first step towards developing a strategy to either significantly reduce or even eliminate the need for lifelong immunosuppression.

Herein, it is demonstrated that Recruitment-MP can 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 proinflammatory 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 [162].

5.2 METHODS

5.2.1 Animals

Six to eight-week-old male Lewis, Brown Norway, and Wistar Furth rats (Charles River Laboratories, Wilmington, MA) were used. Animals were maintained under an Institutional Animal Care and Use Committee (IACUC) protocol in a specific pathogen-free environment at the University of Pittsburgh.

5.2.2 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.

5.2.3 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 described (Figure 5). 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 four 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).

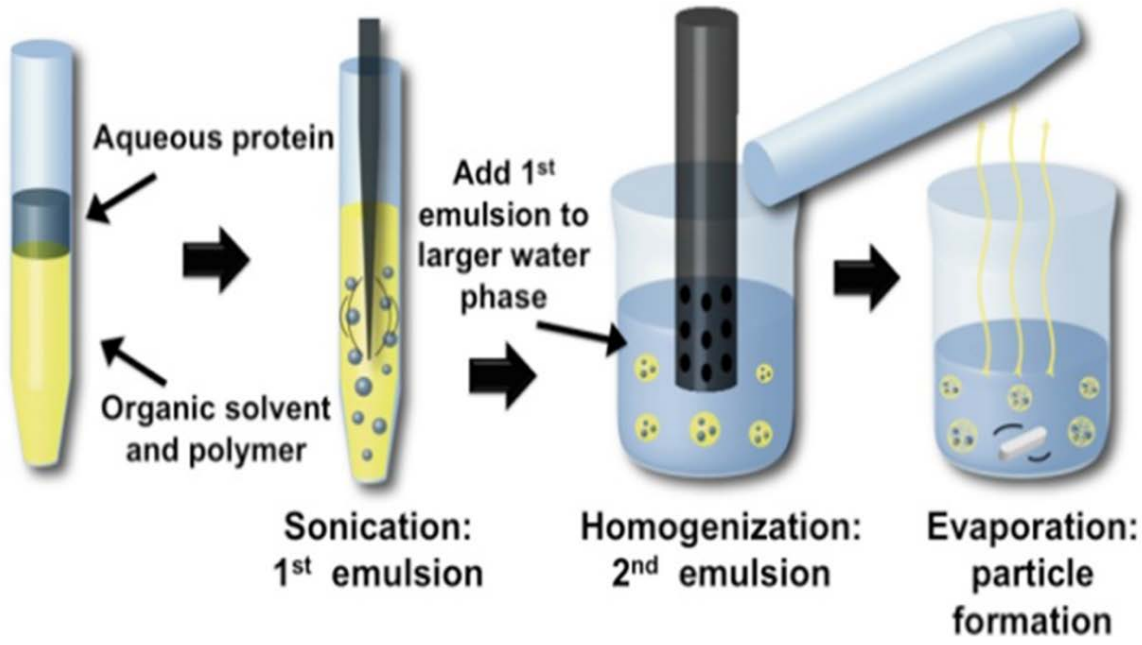

Figure 5. Recruitment-MP were fabricated using a water in oil in water (W/O/W) double emulsion technique.

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 6 below; microspheres were resuspended in 1 mL of fresh PBS and returned to the rotator at 37°C.

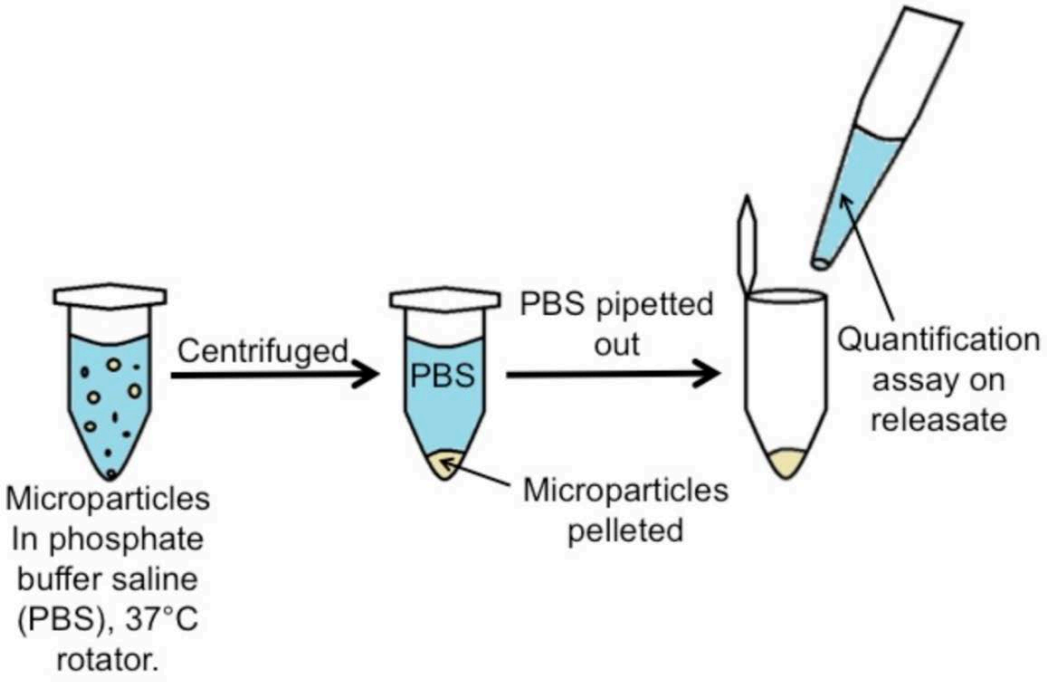

Figure 6. *In vitro* release of CCL22 from Recruitment-MP was measured for 40 days.

5.2.4 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 7. 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).

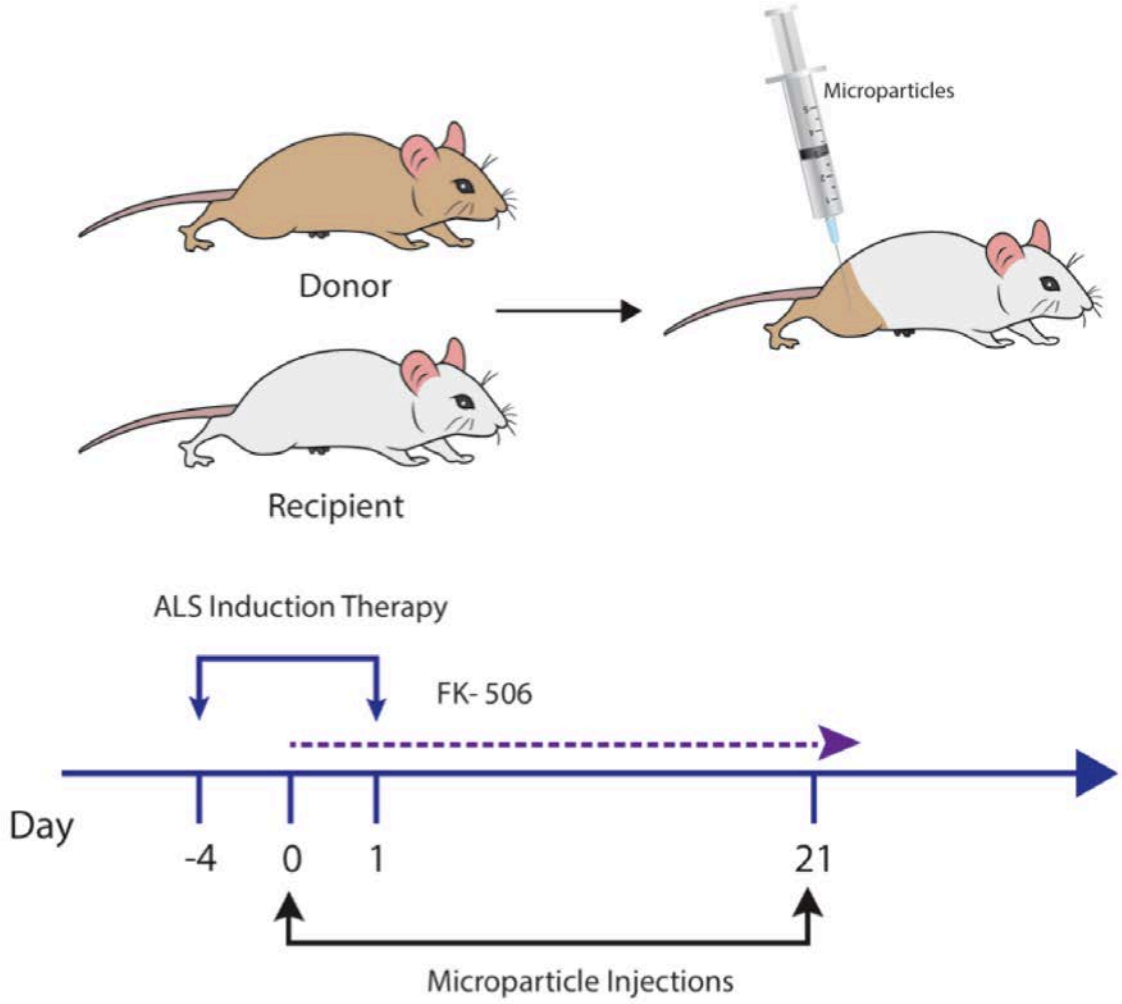

Figure 7. The experimental timeline for all animals receiving hind limb VCA. All animals receive the same baseline immunosuppression protocol consisting of 21 days of 0.5 mg/kg FK506 IP as well as two 0.5cc IP doses of rabbit anti-rat lymphocyte serum (ALS). Animals receiving microparticles were administered microparticles subcutaneously in the transplanted graft (unless otherwise noted) on postoperative days (POD) 0 and 21.

5.2.5 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 8.

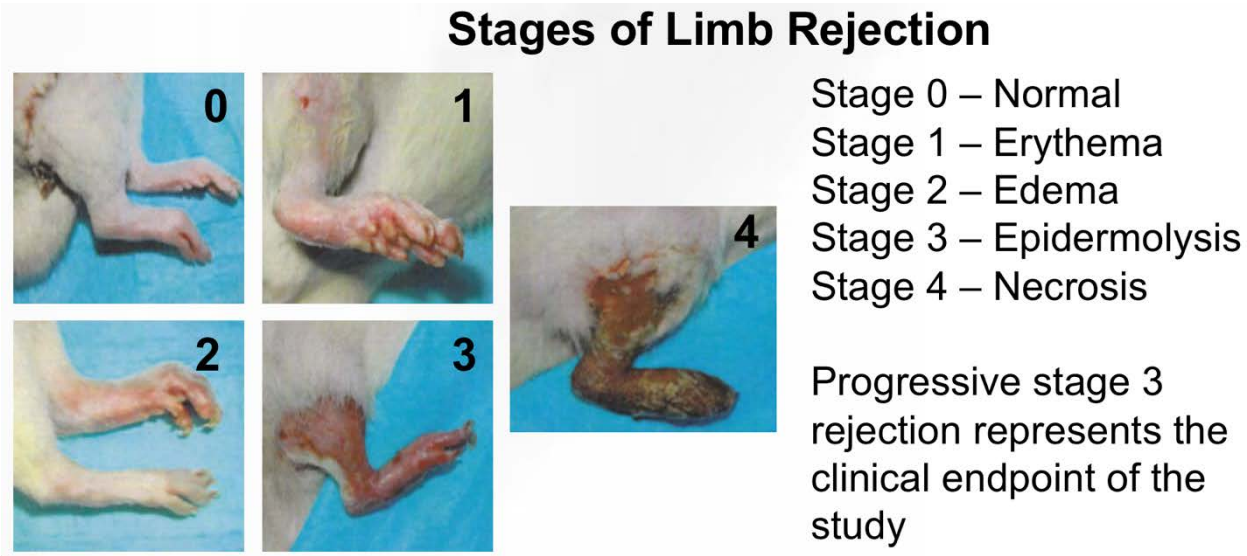

Figure 8. The clinical stages of limb rejection. Animals receiving a hind limb VCA were monitored daily and scored on a five-point rejection scale. Limbs displaying progressive stage 3 rejection were considered "rejected."

5.2.6 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.

5.2.7 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).

5.2.8 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.

5.2.9 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. $CD4^+CD25^-$ (Teff) and $CD4^+CD25^{hi}$ (Treg) populations were sorted using a fluorescence-activated cell sorter. To assess proliferative function, $CD4^+CD25^-$ (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:

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

5.2.10 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 9. 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.

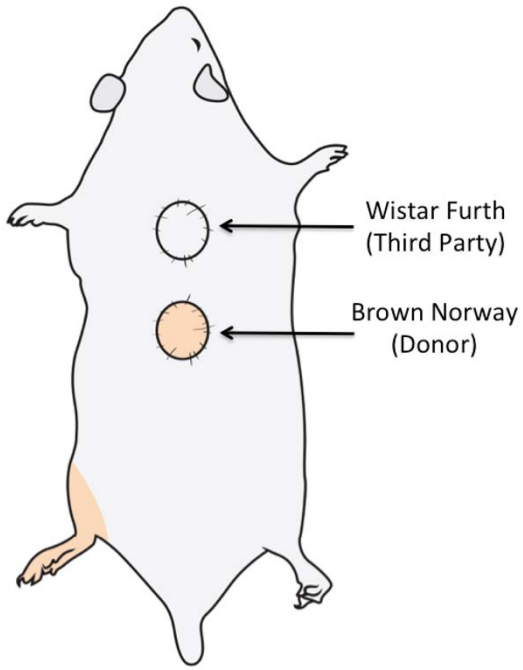

Figure 9. In vivo donor antigen-specific tolerance was tested using a secondary skin graft challenge to animals with long-term surviving hind limb VCAs.

5.2.11 Statistics

All data are 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.

5.3 RESULTS

5.3.1 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 10C demonstrates that Recruitment-MP can 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 10A). The surface of Recruitment MP was specifically formulated to be porous, to allow continuous release (without periods of lag) of chemokine (Figure 10A), 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 the vascular endothelium, with consequent immobilization at the site of placement (Figure 10B).

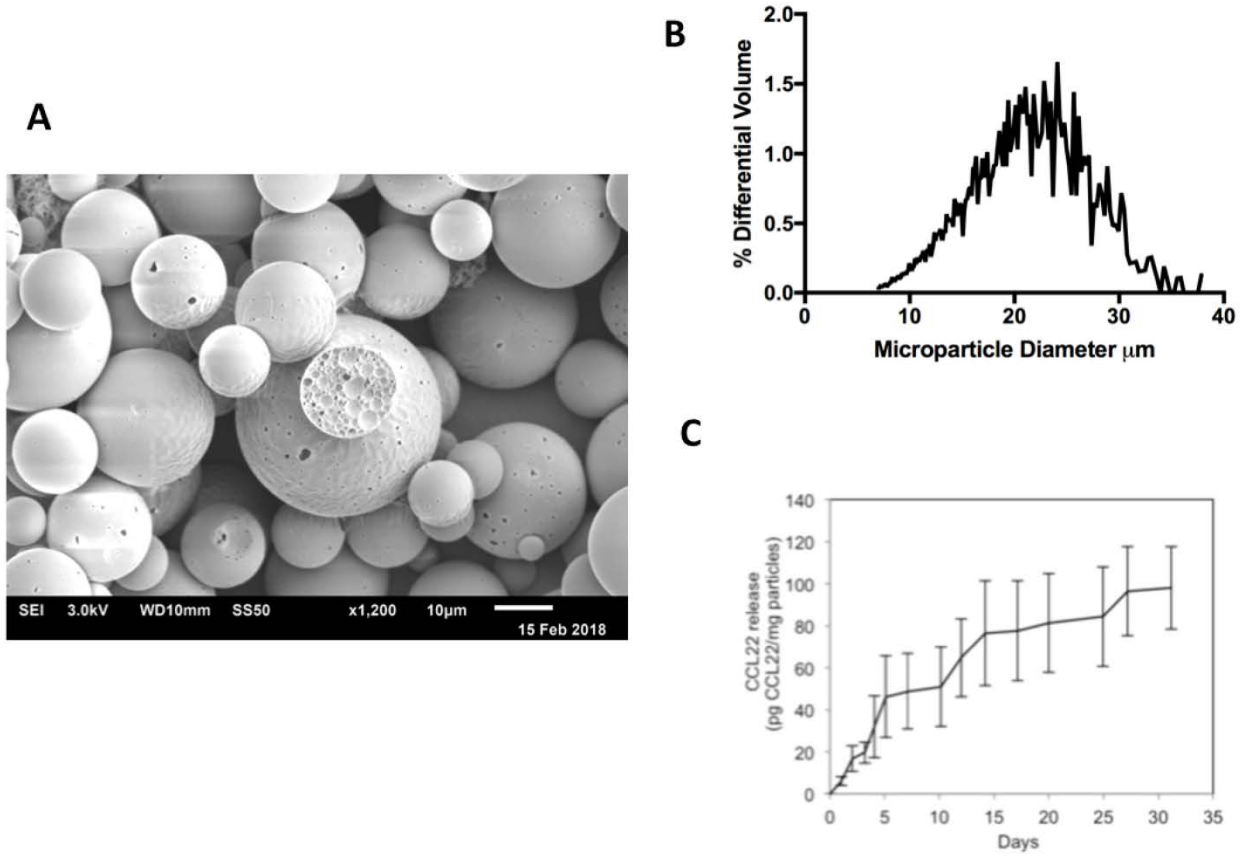

Figure 10. The characterization of Recruitment-MP. Recruitment-MP is spherical in shape and slightly porous (A) with an average particle diameter of 18.3µm (B). Release kinetics demonstrate that Recruitment-MP release CCL22 in a linear fashion for 30 days (C).

5.3.2 Recruitment-MP prevents rejection and promotes 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). Rejection was defined by grafts displaying signs of progressive Grade III rejection were considered.

Animals only receiving the baseline immunosuppression protocol of FK506/ALS served as our basis for comparison. As can be seen in Figure 11A 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 an effective gradient necessary for recruiting Tregs and prolonging graft survival. As illustrated in Figure 11A, 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 >200 days 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 significantly prolonged graft survival when compared to animals only receiving the baseline immunosuppression protocol.

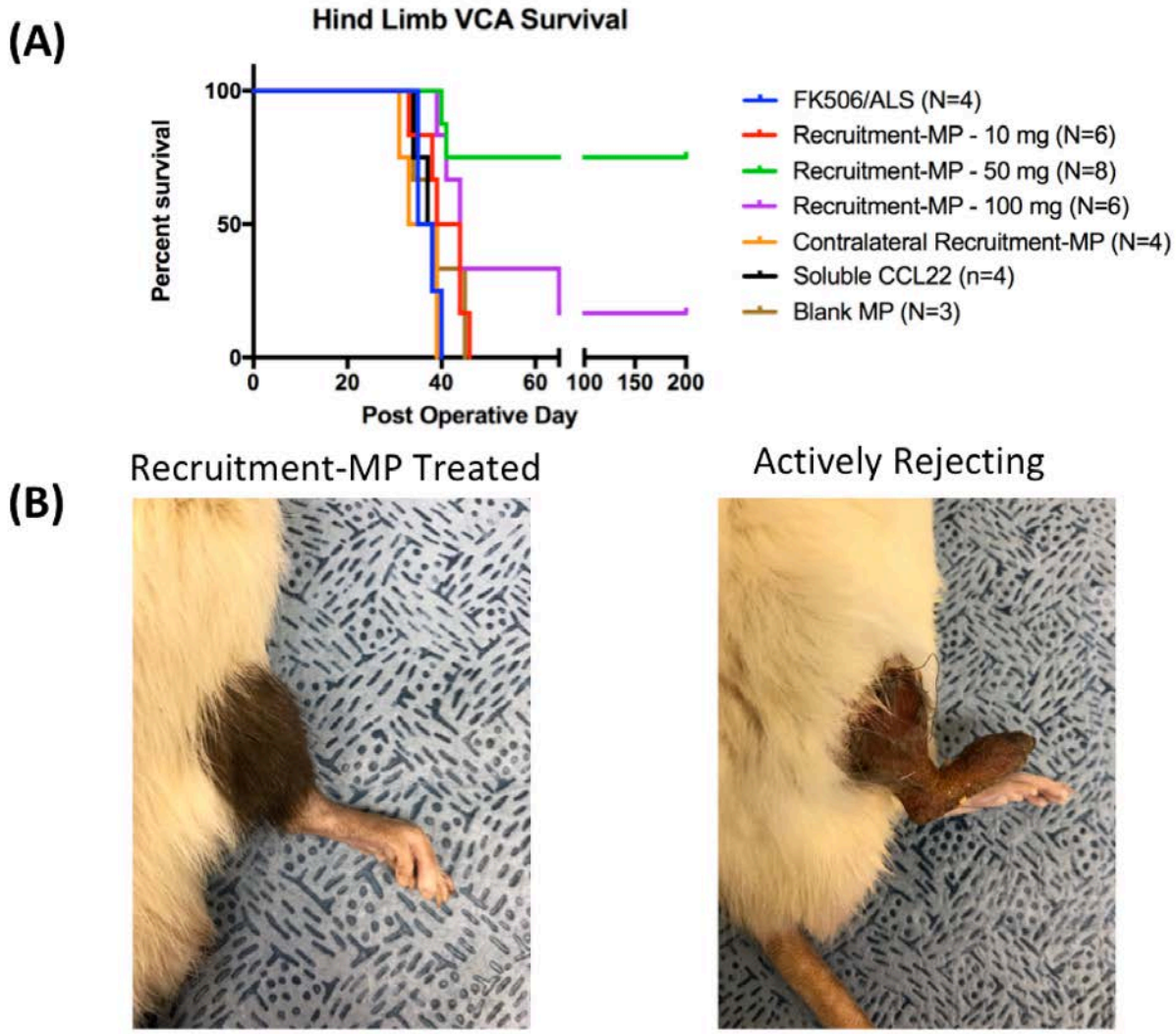

Figure 11. Treatment with 50mg of Recruitment-MP is able to prolong allograft survival indefinitely in 6/8 animals. These results are statistically significant at $p<0.05$ when compared to all other groups (A).

Histologically, grafts undergoing Grade III-IV rejection showed complete obliteration of the epidermis, substantial mononuclear infiltration in the dermis and perivascular regions (Figure 12). 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 12).

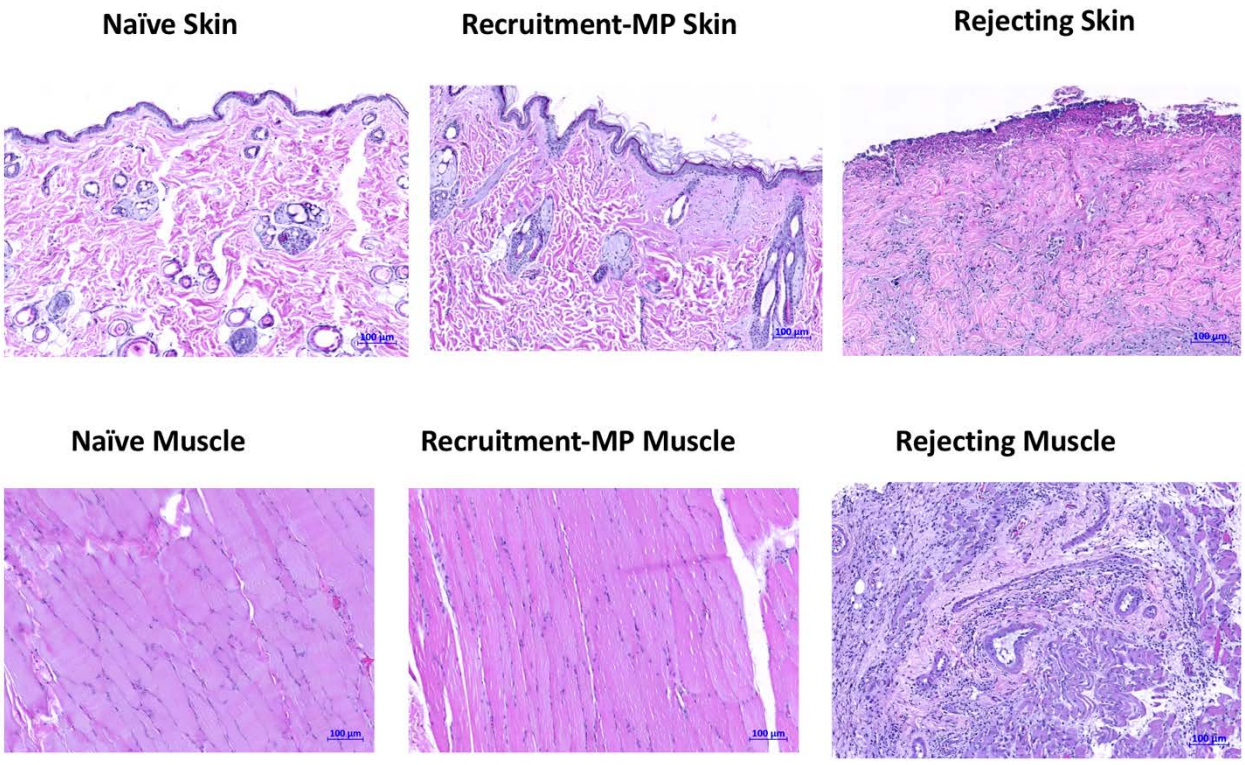

Figure 12. Recruitment-MP is able to preserve the architectural integrity of intra-graft tissues (muscle and skin). Tissue samples were taken from rejecting animals display complete destruction of tissue architecture with dense mononuclear infiltration (right panel).

5.3.3 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 the experimental endpoint, POD >200). We then measured the 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 13). 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.

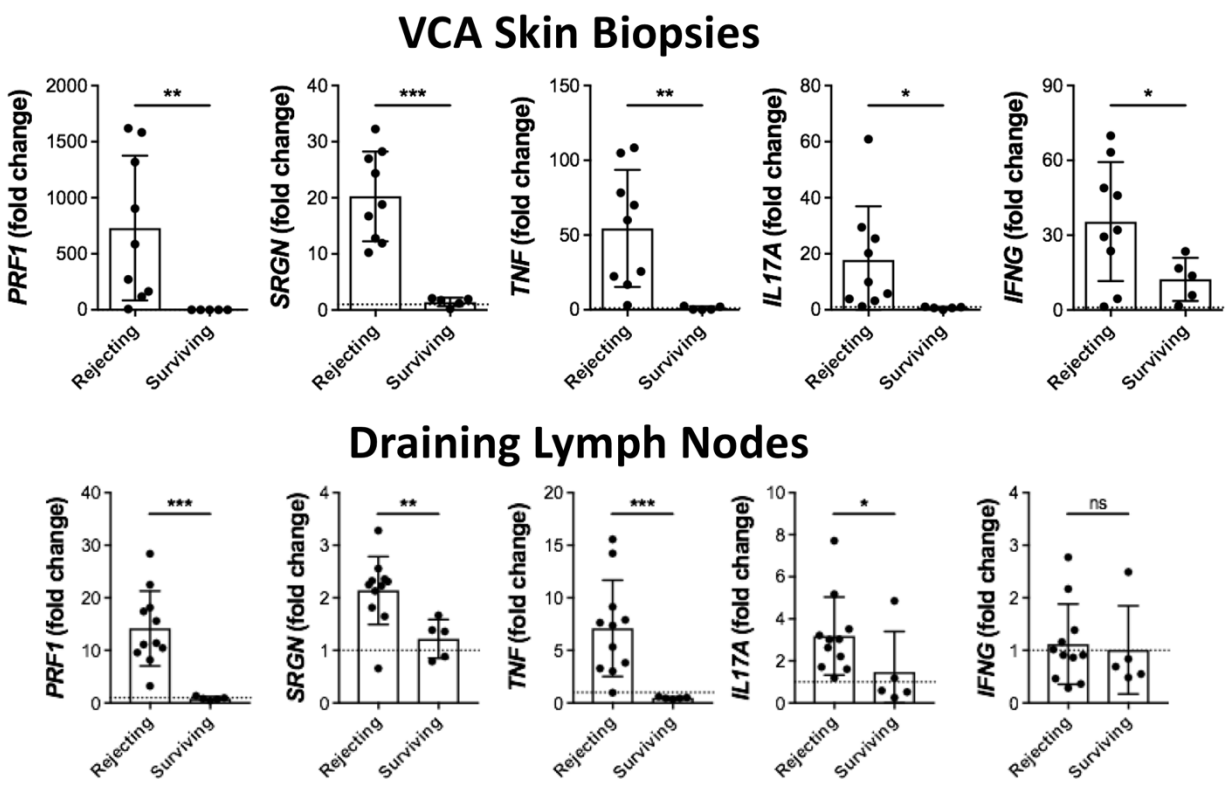

Figure 13. Relative mRNA expression in skin samples and draining lymph nodes from rejecting hind limb allografts (n=9 for skin, n=11 for draining lymph nodes) vs. surviving Recruitment-MP treated hind limb allografts (n=5 for both skin and lymph nodes). Expression levels are presented as fold changes ($2^{-\Delta\Delta Ct}$) 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 *** $p < 0.001$.

5.3.4 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 $CD4^+CD25^{hi}$ $FoxP3^+$ 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 14A). However, allograft draining and non-draining lymph nodes from Recruitment-MP treated animals showed a significantly decreased percentage of inflammatory $CD4^+$ $IFN\gamma^+$ 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 $CD4^+$ $IFN\gamma^+$ cells than from non-draining lymph nodes of actively rejecting animals (Figure 14B).

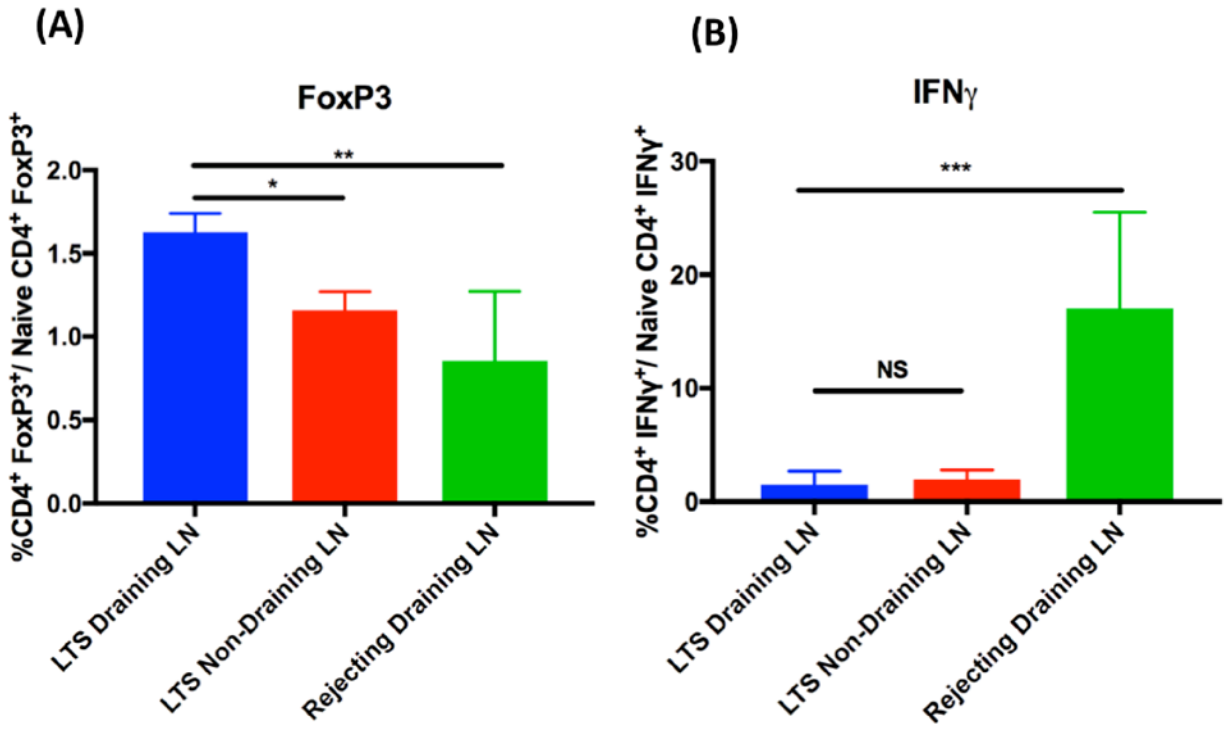

Figure 14. Phenotypic analysis of CD4+ cells in draining and non-draining lymph nodes of long-term surviving grafts treated with 50mg Recruitment-MP (LTS, n=4) and draining lymph nodes of actively rejecting controls (n=3). Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

5.3.5 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: $CD4^{+}CD25^{hi}$ (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 15A, 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 15B).

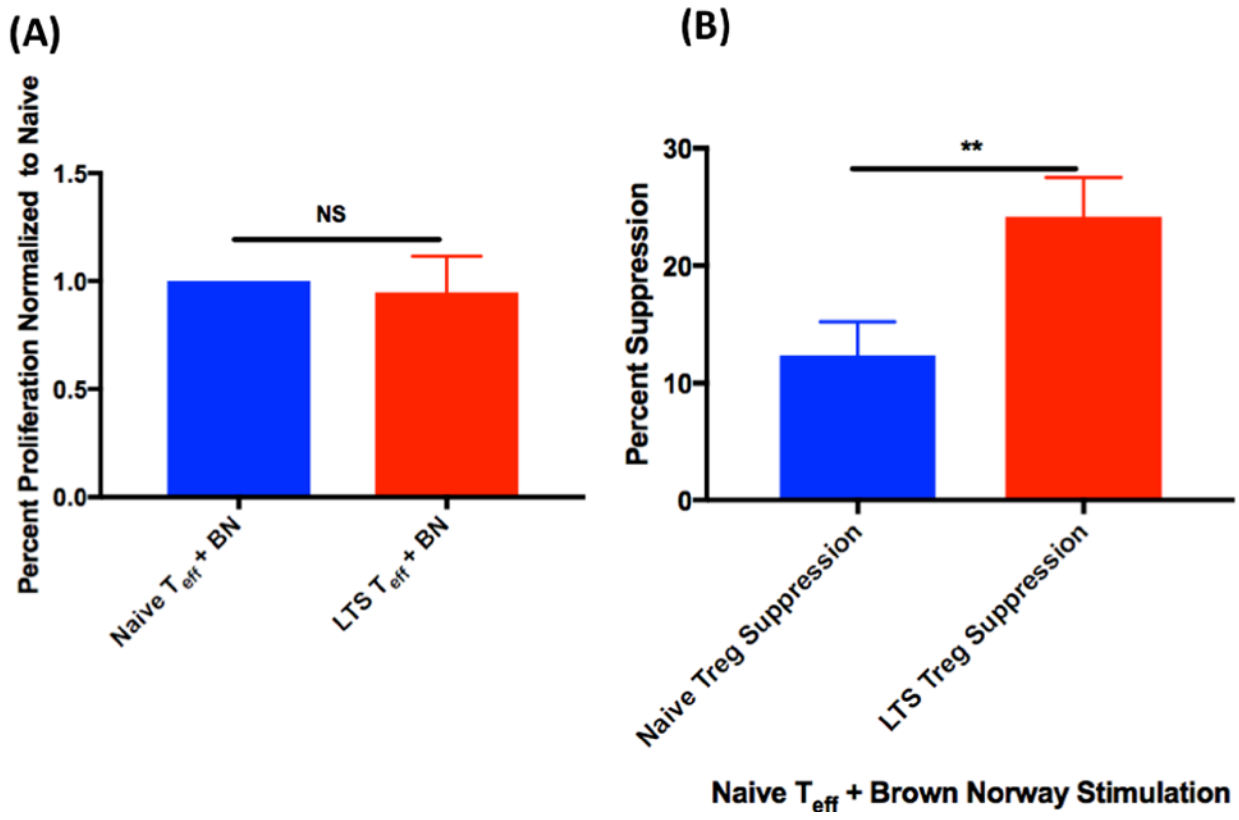

Figure 15. Functional analysis of T cells isolated from animals with grafts treated with 50mg Recruitment-MP (LTS). There is no significant difference in proliferative capacity of $CD4^+CD25^-$ Teff (stimulated with Brown Norway (BN) splenocytes) isolated from animals treated with Recruitment compared with $CD4^+CD25^-$ Teff (stimulated with BN splenocytes) isolated from naïve Lewis rats (A). The percent proliferation was normalized to navie T_{eff} proliferation with BN stimulation. $CD4^+CD25^{hi}$ Tregs isolated from Recruitment-MP treated animals are more effective at suppressing BN mediated Naïve Teff proliferation than $CD4^+CD25^{hi}$ Tregs isolated from naïve Lewis rats (B). Significant differences are indicated by ** $p < 0.01$.

5.3.6 Tregs from Recruitment-MP treated animals exhibit donor specific suppression of Teff proliferation.

To test the donor antigen specificity of $CD4^{+}CD25^{hi}$ Tregs isolated from Recruitment-MP treated VCA recipients, yet another MLR was set up. Naïve Lewis $CD4^{+}CD25^{-}$ Teff were co-cultured with $CD4^{+}CD25^{hi}$ 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 16 A).

5.3.7 Recruitment-MP treatment confers systemic donor-specific tolerance to hind-limb recipients in vivo.

To test whether Recruitment-MP can 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 Xeroform 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 16 B-C).

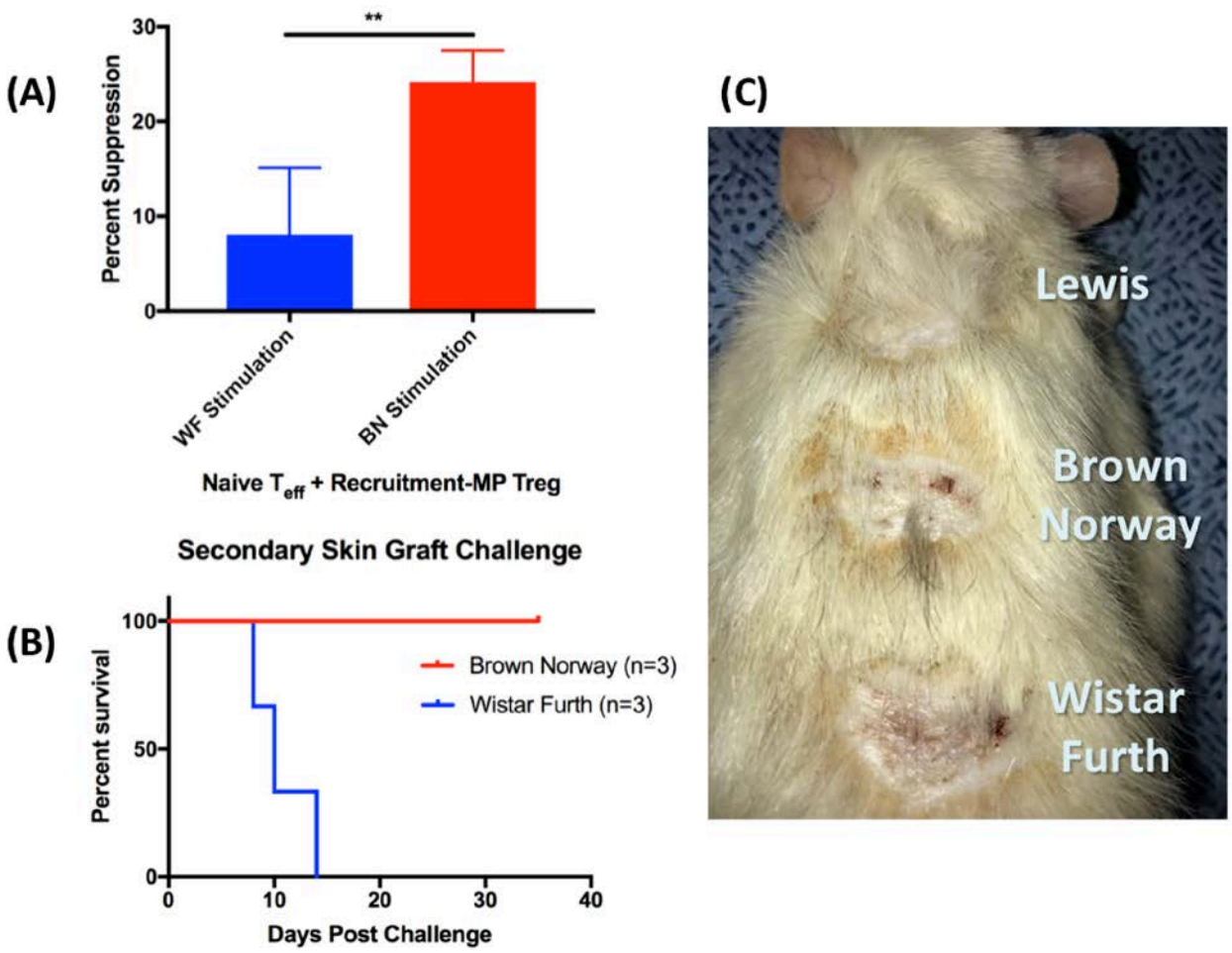

Figure 16. Recruitment-MP is able to confer donor antigen-specific tolerance to animals with long-term surviving grafts. $CD4^{+}CD25^{hi}$ Tregs isolated from Recruitment-MP treated animals were co-cultured with $CD4^{+}CD25^{-}$ Teff isolated from naïve Lewis rats and then subject to stimulation with either Brown Norway (BN) or Wistar Furth (WF) splenocytes. Tregs isolated form Recruitment-MP treated animals are more effective at suppressing BN mediated proliferation than WF mediated proliferation (A). To demonstrate donor antigen-specific tolerance *in vivo*, Recruitment-MP treated animals with long-term surviving grafts were challenged with full thickness 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).

5.4 DISCUSSION

The most significant issue facing the field of transplant immunology is graft loss due to allorecognition and acute rejection, necessitating the need for transplant recipients to be placed on lifelong, high dose, toxic immunosuppression. This matter is further exacerbated in the emerging field of VCA, as grafts are not viewed as life-saving interventions (but rather life-giving), raising ethical issues concerning the risk/reward of such procedures. As such, to allow for the routine and widespread use of VCA, it is of paramount importance to develop alternative strategies to limit or even eliminate the need for chronic systemic immunosuppression.

In the past decade, mounting evidence has accumulated implicating a pivotal role for Tregs in the prevention of allograft rejection and induction of tolerance [64, 72, 167]. Multiple groups have demonstrated that adoptive transfer of ex vivo processed Tregs can restore homeostasis in various preclinical models of inflammation and, starting in 2009; multiple smaller trials demonstrated the safety and efficacy of Treg cell therapy in humans for multiple pathologies [75-77, 168-170]. Though promising in experimental models and small trials, the ubiquitous clinical implementation of these approaches is faced with several logistical and regulatory hurdles including (but not limited to) issues related to cGMP isolation of Tregs and maintaining the stability of adoptively transferred Tregs [70, 81, 82, 171].

Given the significant challenges with ex vivo expanded Treg therapies, it is desirable to develop an alternative, cell-free strategy to harness the immunoregulatory potential of Tregs. It is recognized that certain cancerous tumors secrete the Treg recruiting chemokine CCL22 as a means to create a pro-regulatory environment in the tumor milieu [165]. Further, previous attempts to mimic this phenomenon involved the use of an adenovirus to induce expression of CCL22 in a

mouse model of autoimmune diabetes [141]. Herein, we describe a strategy to locally recruit Tregs to the site of a VCA using purely synthetic, CCL22 releasing microspheres (Recruitment-MP).

A rat hind-limb model of VCA was employed, using Brown Norway rats as donors and Lewis rats as recipients. All animals (in each group) received the same short course baseline immunosuppression protocol consisting of two doses of ALS induction therapy and 21 days of tacrolimus maintenance therapy. Our rationale for including a short course of immunosuppression was twofold: 1.) to ensure that Recruitment-MP was given enough time to establish an adequate gradient of CCL22 and 2.) to demonstrate that Recruitment-MP can be used with an immunosuppression protocol that resembles those used clinically [160].

Because the effectiveness of Recruitment-MP hinges upon the generation of a gradient of CCL22 to recruit Tregs, three doses (9mg, 50mg, and 100mg) of Recruitment-MP was tested to determine an effective formulation for sufficient directional migration of Tregs to the transplanted limb. If the concentration of CCL22 is too high, chemokine receptor binding could become saturated (ultimately inhibiting chemotaxis), conversely, if the concentration of CCL22 is too low, then it may not be possible to create a gradient capable of affecting Tregs distant from the site of placement [172].

The data suggest that treatment with only two doses of 50mg of Recruitment-MP (at POD 0 and 21) can significantly prolong hind-limb allograft survival when compared to the 9mg and 100mg doses which do not show any statistical significance when compared to animals only receiving FK506/ALS (Figure 11A). Furthermore, it also demonstrated the necessity of a release system, as a subcutaneous bolus injection of CCL22 is also not able to prolong graft survival (Figure 11A). Finally, it should be noted that the tolerogenic effects seen with Recruitment-MP

are due to local release of only nanogram quantities CCL22 per day, as opposed to conventional immunosuppression which requires milligrams of drug per day.

Flow cytometric analysis of lymph nodes from long-term surviving Recruitment-MP VCA recipients demonstrated a significantly higher percentage of $CD4^{+}CD25^{hi}FoxP3^{+}$ cells in the draining (ipsilateral) lymph nodes than in the non-draining (contralateral) lymph nodes as well as draining lymph nodes from rejecting animals (Figure 14A). This result further supports our hypothesis that Recruitment-MP can promote VCA tolerance via local enrichment of Tregs via chemotactic recruitment. Furthermore, increasing percentages of functional Tregs also lead to a concomitant decrease in the percentage of $CD4^{+}IFN^{+}$ cells in draining lymph nodes of long-term surviving lymph nodes and an increase in $CD4^{+}IFN^{+}$ cells in draining lymph nodes of rejecting animals (Figure 14B). In addition to reducing inflammation at the cellular level, Recruitment-MP was also able to substantially reduce inflammation at the RNA level in both intragraft cutaneous samples and in draining lymph nodes. mRNA expression of TNF, Perforin-1, Serglycin, and IL-17 was decreased in draining lymph nodes and cutaneous intragraft skin biopsies of long-term survivors when compared to actively rejecting animals (Figure 13). This result is unsurprising as Tregs are known to inhibit Th1/Th17 T helper cells and their associated cytokines [64, 173-175]. Taken together, results at the macroscopic, microscopic, and genomic and cellular level tend to indicate that Recruitment-MP can achieve graft hypo-responsiveness via local enrichment of suppressive Tregs. Importantly Recruitment-MP functions by enriching for regulatory cells responsible for establishing and maintaining homeostasis, as opposed to traditional immunosuppression which prevents allograft rejection by globally impairing the ability of CD4+ helper T cells to mount an inflammatory response.

Additionally, Tregs were further implicated as an essential component to the observed tolerance exhibited by long-term survivors using a series of MLRs. Importantly, it was shown that Treg depleted $CD4^{+}CD25^{-}$ Teff from long-term survivors showed no significant immune hypo-responsiveness toward donor stimulation when compared to naïve $CD4^{+}CD25^{-}$ Teff (Figure 15A), suggesting that $CD4^{+}CD25^{hi}$ Tregs (from long-term survivors) are what is contributing to the observed hypo-responsiveness. Furthermore, it was also demonstrated that $CD4^{+}CD25^{hi}$ Tregs isolated from long-term survivors were more effective at suppressing donor antigen proliferation than $CD4^{+}CD25^{hi}$ Tregs from naïve animals, suggesting Recruitment-MP may have imparted donor specific suppressive function to treated VCA recipients with long-term surviving grafts (Figure 15B). To explore this even further, it was next demonstrated that $CD4^{+}CD25^{hi}$ Tregs from long-term survivors were more effective at suppressing Brown Norway (donor) mediated proliferation than Wistar Furth (third party), even further suggesting an antigen-specific role for Tregs isolated from Recruitment-MP treated recipients (Figure 16A). As a final step to demonstrate donor specificity, and that it was not limited to in vitro assays, full-thickness skin grafts were grafted from both Brown Norway (donor), and Wistar Furth (third party) recipients to Recruitment-MP treated rats with long-term surviving grafts. As shown in Figure 16B-C, this in vivo test of antigen specificity mirrored results shown in vitro, with all animals accepting donor skin grafts and failing to accept third-party skin grafts.

While the data presented herein strongly suggest that Recruitment-MP can establish dominant antigen tolerance in rodents receiving a hind-limb transplant, there are several limitations to this study worth noting. For example, the model employed in this work utilized rats as a model species (as opposed to mice) due to exceeding technical difficulties associated with reliably re-anastomosing the blood vessels in mice. As such, this study was very much limited with respect

to the availability of a host of biochemical and genetic tools that are often readily available when working with mice. For instance, the use of a FoxP3EGFP reporter animal would be useful for identifying and isolating Tregs from long-term survivors. Furthermore, there does not currently exist a method to deplete Tregs in rats in vivo that is not cost prohibitive. This would be especially useful to demonstrate that it is possible to break the observed VCA tolerance by depleting Tregs.

In summary, the work described here suggests that subcutaneous administration of Recruitment-MP can prevent hind-limb allograft rejection and promote donor antigen-specific tolerance to the transplanted graft. The observed tolerance is likely due to the local recruitment of Tregs (and a concomitant decrease in pathogenic Th1 and Th17 cells/biomarkers) via a sustained CCL22 gradient established by Recruitment-MP. Further studies will focus on scaling this technology in preparation of large animal preclinical studies and demonstrating the clinical efficacy of Recruitment-MP in models of solid organ transplantation and even autoimmunity.

6.0 TREG INDUCING MICROPARTICLES INDUCE DONOR ANTIGEN SPECIFIC TOLERANCE IN A RAT HIND LIMB TRANSPLANT MODEL

6.1 INTRODUCTION

In the previous chapter, a biomimetic approach to locally recruit Tregs to a point sight *in vivo* was described. Specifically, it was demonstrated that CCL22 releasing microspheres (Recruitment-MP) could prolong rodent hindlimb 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) can induce Tregs via secretion of Treg-trophic factors such as TGF-β, and

IL-2 [176-180]. Also, 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[181]*. Based on this information/inspiration, we have developed local, controlled release systems herein referred to as **Tr**eg-**I**nducing (**TRI**) biodegradable systems that can 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[128]. 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 [128, 182, 183].

Herein, we put forth data that strongly suggests that TRI-MP can promote tolerance and indefinite allograft survival (>300 days) in an aggressive rat hindlimb 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. Ultimately, this strategy has the potential to potentially reduce, or even eliminate, the need for systemic immunosuppression in both VCA and SOT.

6.2 METHODS

6.2.1 Animals

Six to eight-week-old male Lewis, Brown Norway, and Wistar Furth rats (Charles River Laboratories, Wilmington, MA) were used. Animals were maintained under an Institutional Animal Care and Use Committee (IACUC) protocol in a specific pathogen-free environment at the University of Pittsburgh.

6.2.2 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.

6.2.3 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 5). 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 polylactic-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 small lipophilic molecule, Rapa-MP were prepared using the single emulsion-evaporation technique (Figure 17). Specifically, 2 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.

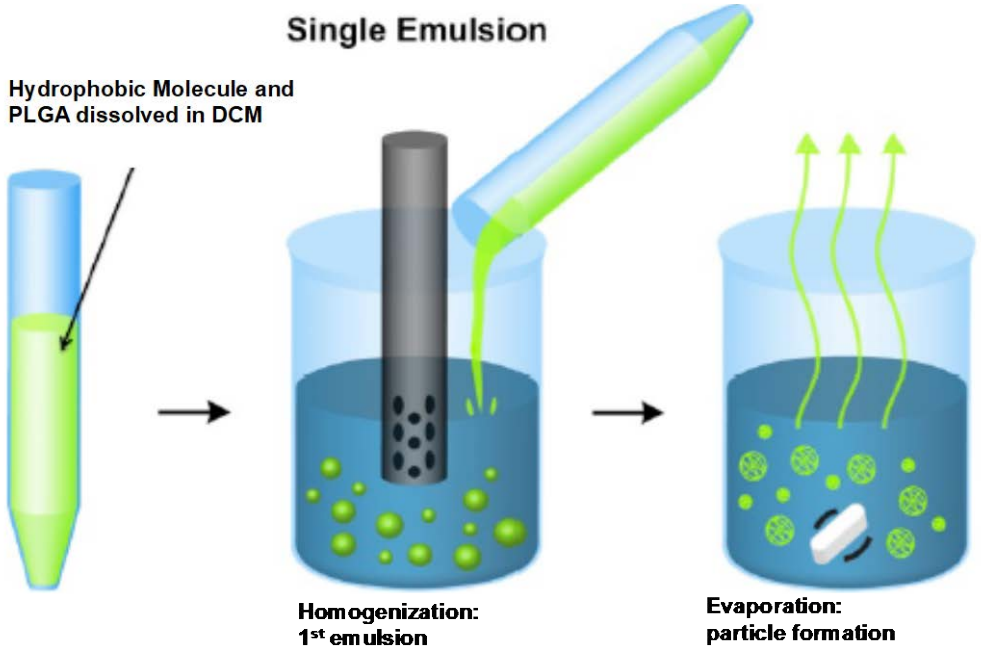

Figure 17. Illustration of the water-in-oil single emulsion technique used to fabricate Rapamycin microparticles (Rapa-MP, a component of TRI-MP).

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 6). 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).

6.2.4 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 7). Microparticles were injected subcutaneously in the lateral aspect transplanted limb (unless otherwise noted) at a 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).

6.2.5 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 8).

6.2.6 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.

6.2.7 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).

6.2.8 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.

6.2.9 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. $CD4^+CD25^-$ (Teff) and $CD4^+CD25^{hi}$ (Treg) populations were sorted using a fluorescence-activated cell sorter. To assess proliferative function, $CD4^+CD25^-$ (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 were 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. $CD4^+CD25^-$ 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. $CD4^+CD25^-$ 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:

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

6.2.10 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 9). 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.

6.2.11 Statistics

All data are expressed as a 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.

6.3 RESULTS

6.3.1 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 18B, 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 18C). Further, we observe a linear release of TGF-β following a ~ 2-week lag phase, and a continuous release from Rapa-MP (Figure 18C, bottom panel).

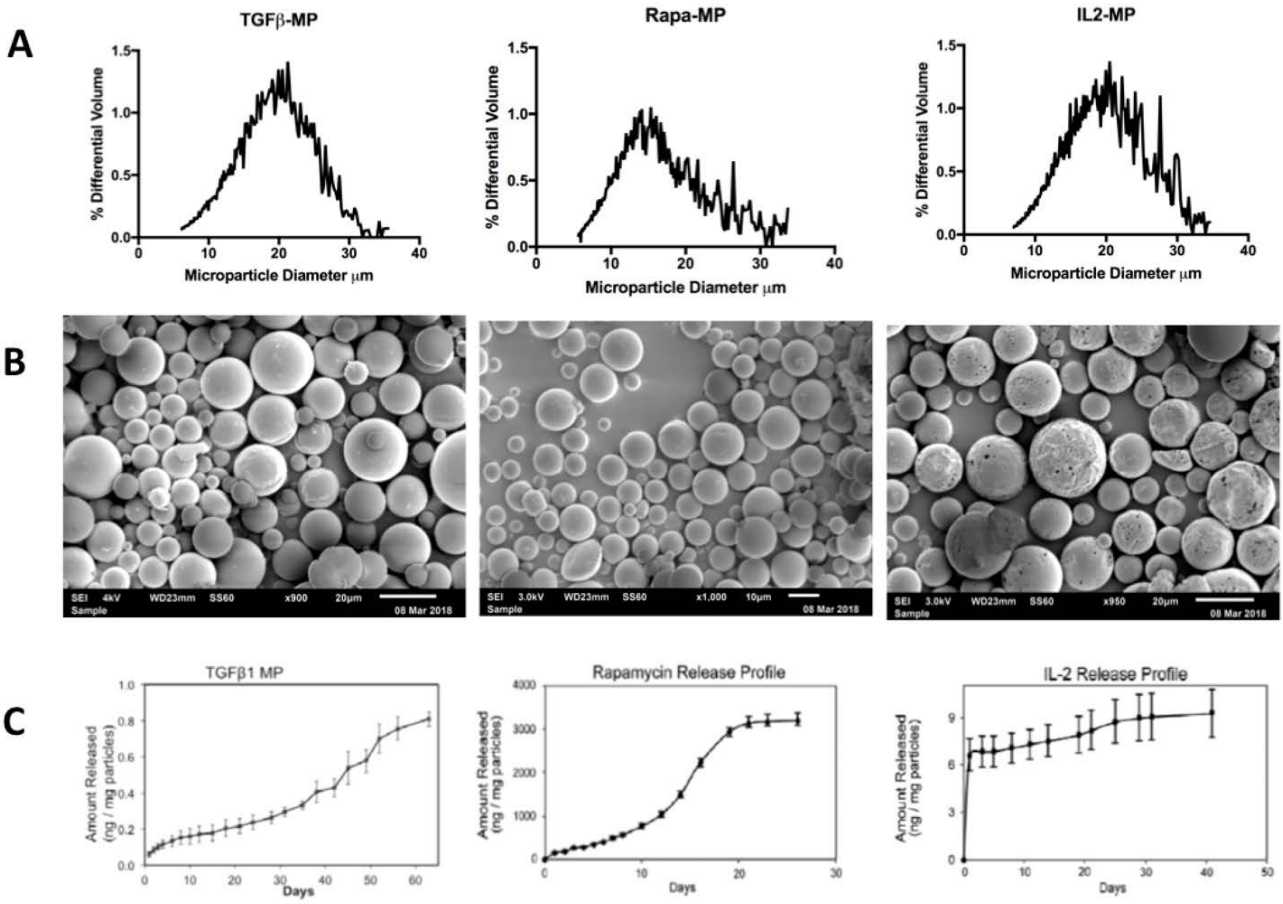

Figure 18. Characterization of TRI-MP. (A) Size distribution of TGFβ-MP, Rapa-MP and IL2-MP. (B) Representative SEM images of TGFβ-MP, Rapa-MP and IL2-MP. (C) Release kinetics of TGFβ-MP, Rapa-MP and IL2-MP (n=3 for each formulation).

6.3.2 Subcutaneous, intragraft treatment with TRI-MP can 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 19B, 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 hindlimb recipients (Figure 19B). 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 19C).

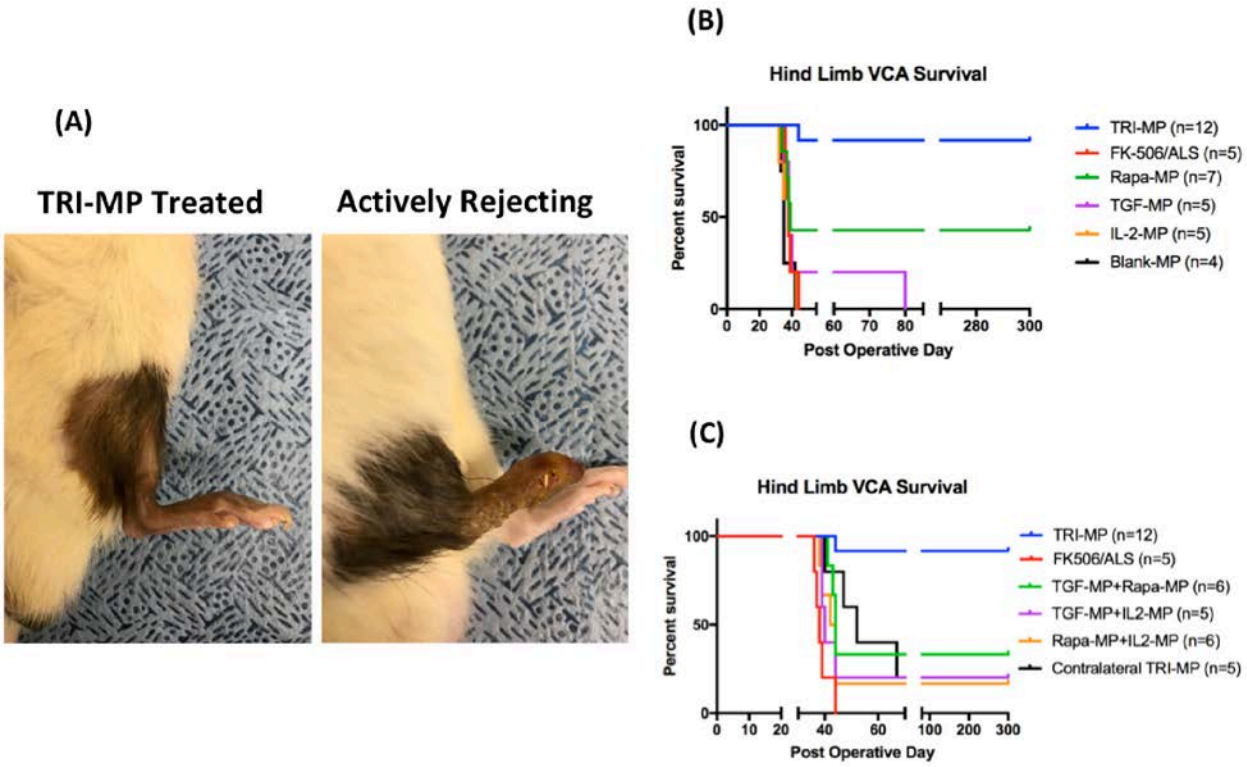

Figure 19. TRI-MP can prevent rejection and promote long-term survival in rodent hind limb recipients. (A) Representative images of TRI-MP treated hind limb (POD>300) showing no signs of rejection and an actively rejecting control. (B) TRI-MP is able to prolong hind limb survival (>300 days in 11/12 animals) in the absence 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 survival ($p<0.001$ for TRI-MP vs. all pairwise controls).

Microscopically, skin grafts 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 20). 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 20).

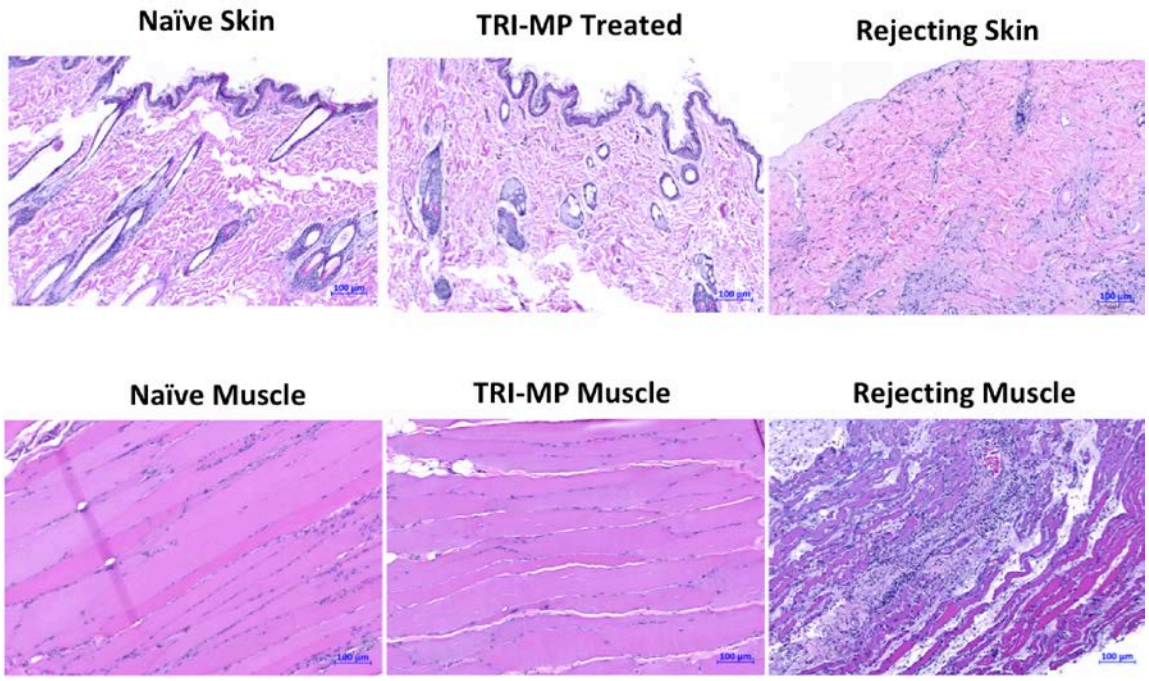

Figure 20. TRI-MP is able to preserve the architectural integrity of intra-graft tissues (muscle and skin). Tissue samples were taken from rejecting animals display complete destruction of tissue architecture with dense mononuclear infiltration (right panel).

6.3.3 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 21 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.

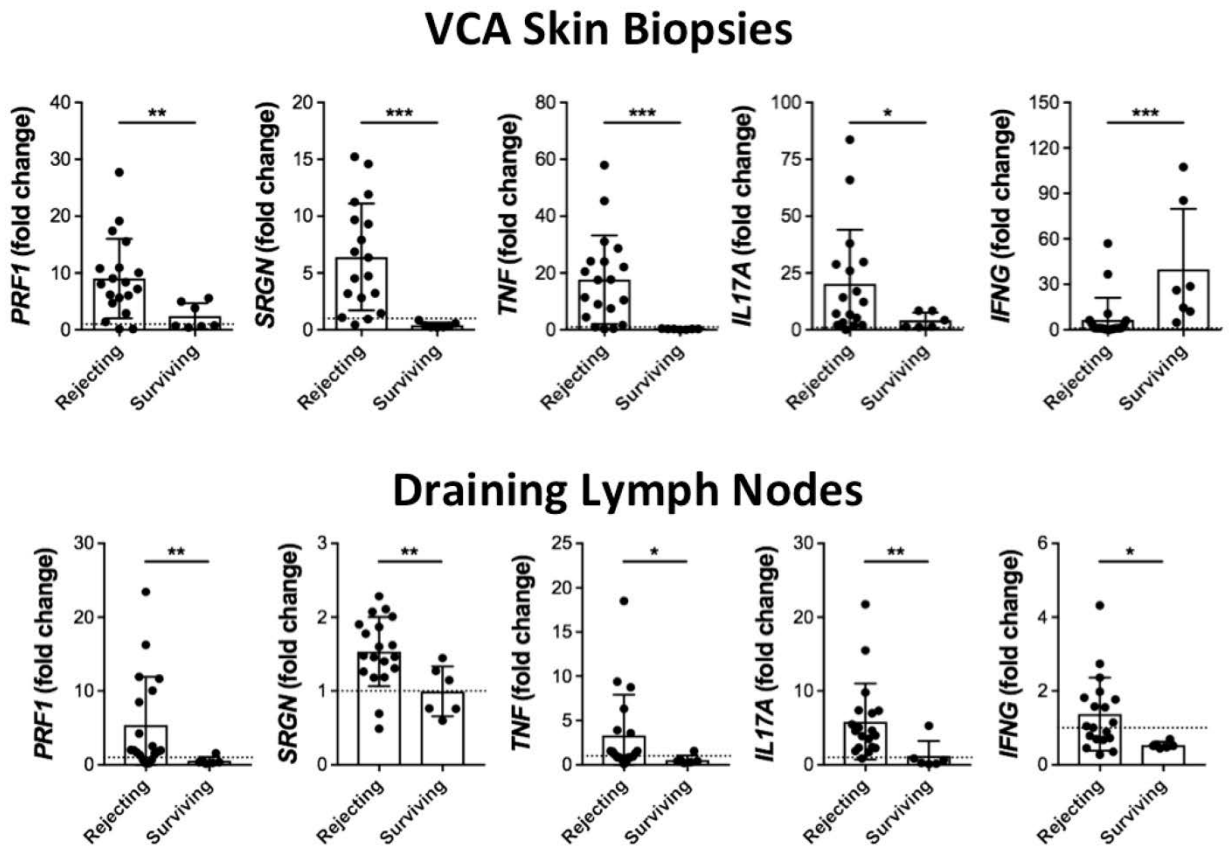

Figure 21. Relative mRNA expression in skin samples and draining lymph nodes from rejecting hind limb allografts (n=17-20 for skin, n=19 for draining lymph nodes) vs. surviving TRI-MP treated hind limb allografts (n=7 for skin and n=6 for lymph nodes). Expression levels are presented as fold changes ($2^{-\Delta\Delta Ct}$) 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 *** $p < 0.001$.

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

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 22A illustrates that (when normalized

to naïve lymph nodes) percentages of $CD4^{+}CD25^{hi}FoxP3^{+}$ 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 $CD4^{+}IFN\gamma^{+}$ cells were significantly higher in draining lymph nodes of actively rejecting animals when compared to TRI-MP treated animals (Figure 22B).

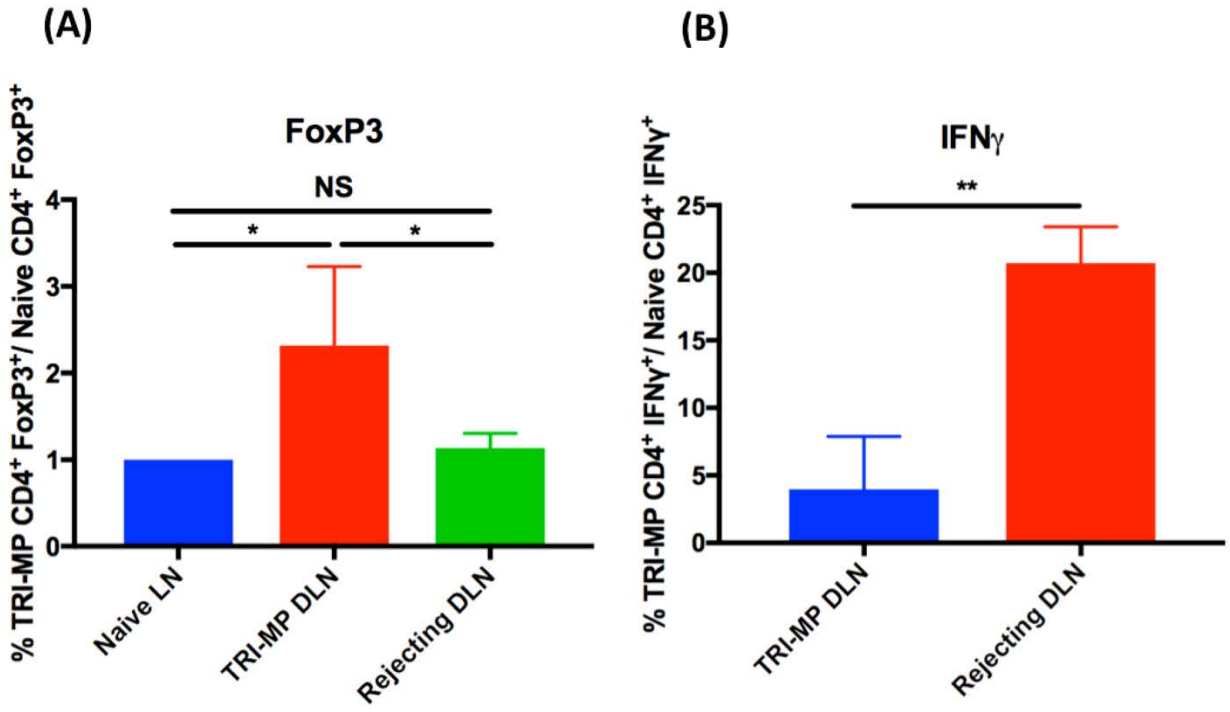

Figure 22. Phenotypic analysis of CD4+ cells in draining and non-draining lymph nodes of TRI-MP treated, long-term surviving grafts (LTS, n=4) and draining lymph nodes of rejecting controls (n=3). Percentages of $CD4^{+}FoxP3^{+}$ and $CD4+IFN\gamma^{+}$ cells in TRI-MP treated and Rejecting animals 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 ** $p < 0.01$.

6.3.5 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 ($CD4^{+}CD25^{hi}$) and Teff ($CD4^{+}CD25^{-}$) populations. As shown in Figure 23A 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.

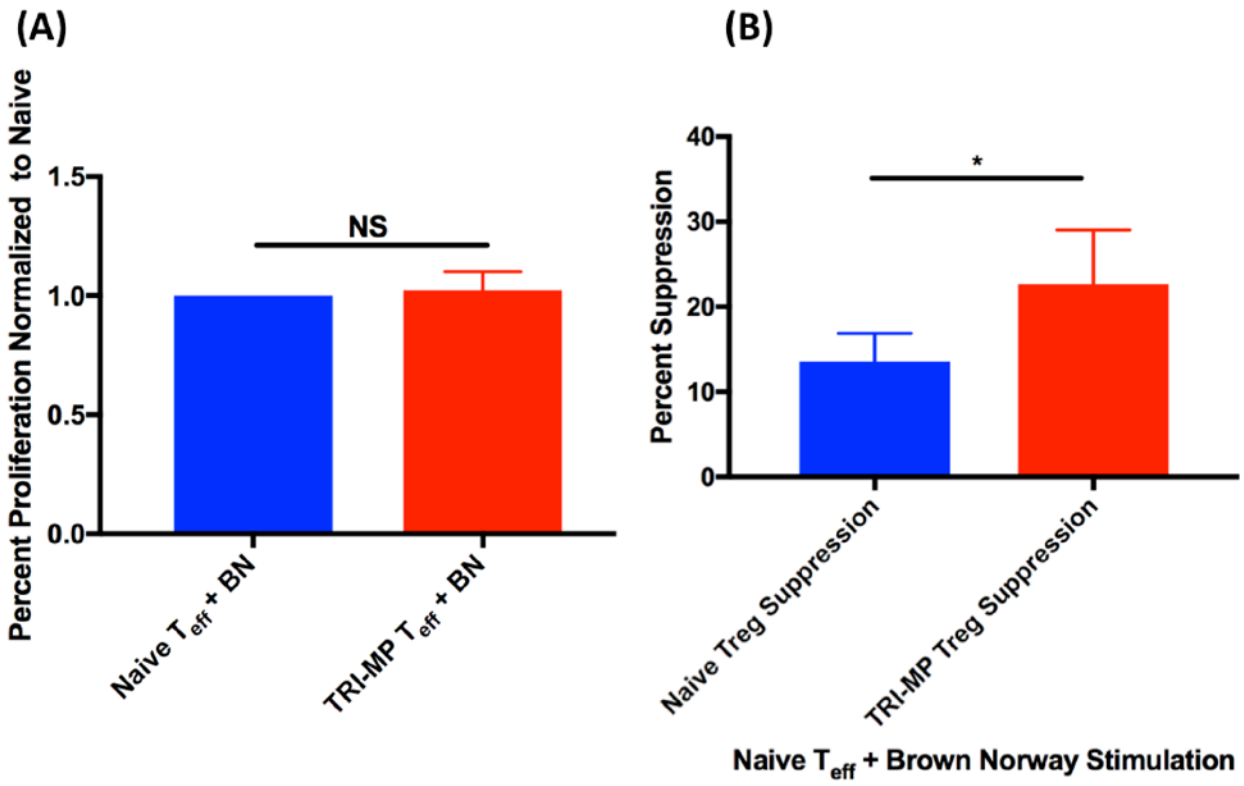

Figure 23. Functional analysis of T cells isolated from animals with TRI-MP treated grafts. (A) There is no significant difference in proliferative capacity of $CD4^{+}CD25^{-}$ Teff (stimulated with Brown Norway (BN) splenocytes) isolated from animals treated with TRI-MP compared with $CD4^{+}CD25^{-}$ Teff (stimulated with BN splenocytes) isolated from naïve Lewis rats. The percent proliferation was normalized to navie T_{eff} proliferation with BN stimulation. (B) $CD4^{+}CD25^{hi}$ Tregs isolated from TRI-MP treated animals are more effective at suppressing BN mediated Naïve Teff proliferation than $CD4^{+}CD25^{hi}$ Tregs isolated from naïve Lewis rats. Significant differences are indicated by * $p < 0.05$

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

To assess functional abilities, $CD4^{+}CD25^{hi}$ 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 23B 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, $CD4^{+}CD25^{hi}$ Tregs isolated from TRI-MP treated rats were tested for 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 24A 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.

6.3.7 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 24B-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.

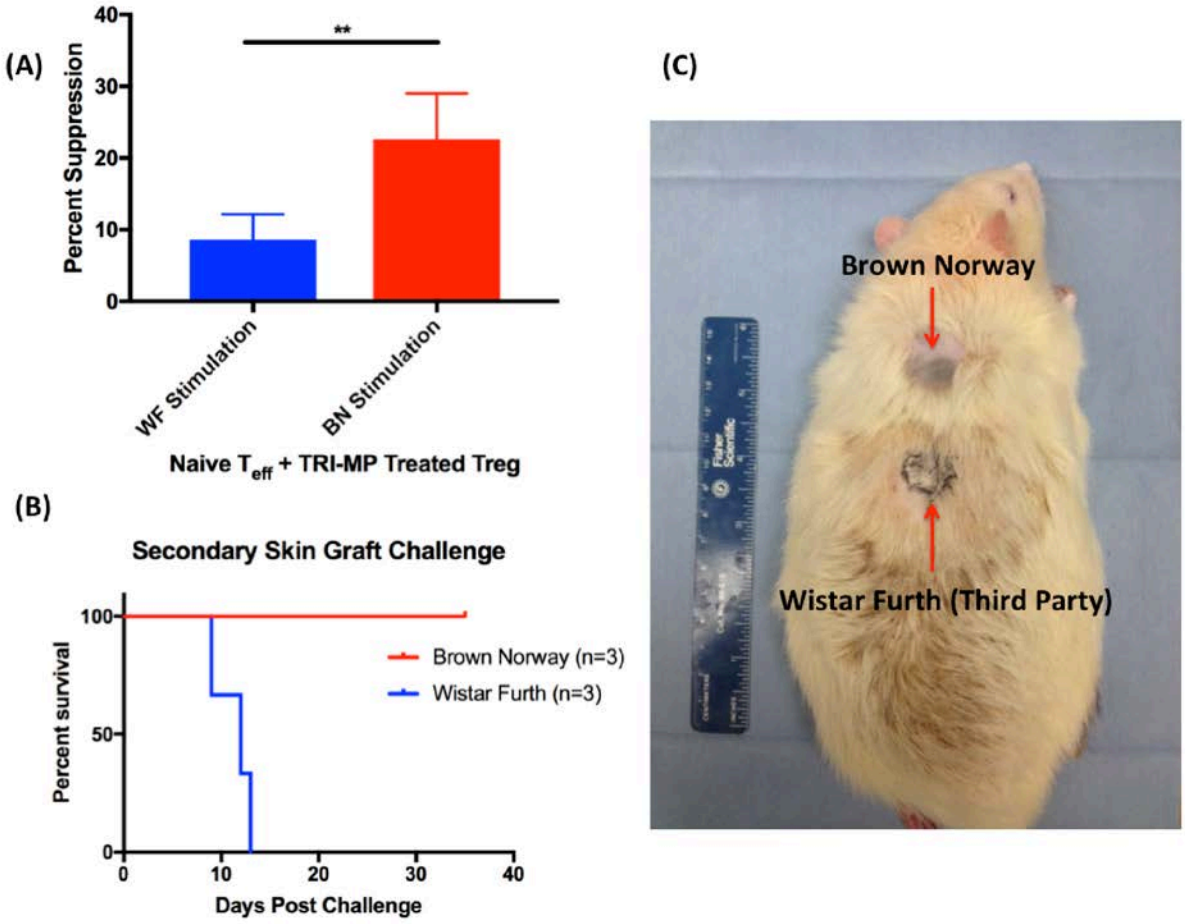

Figure 24. TRI-MP imparts antigen-specific tolerance to rat hindlimb recipients. (A) $CD4^{+}CD25^{hi}$ Tregs isolated from TRI-MP treated animals were co-cultured with $CD4^{+}CD25^{-}$ Teff isolated from naïve Lewis rats and then subject to stimulation with either Brown Norway (BN) or Wistar Furth (WF) splenocytes. Tregs isolated form TRI-MP treated animals are more effective at suppressing BN mediated proliferation than WF mediated proliferation. To demonstrate donor antigen-specific tolerance *in vivo*, TRI-MP treated animals with long-term surviving grafts were challenged with full thickness 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).

6.4 DISCUSSION

The “holy grail” of allotransplantation has been (and continues to be) the induction of robust allograft tolerance in the absence of systemic immunosuppression[184]. Indeed, the concept of allotransplantation has evolved from a miraculous, even divine concept initially only described in ancient Chinese and early Catholic writings to a ubiquitous and life-saving procedure in 21st-century medicine. Still, the need for lifelong, high dose immunosuppression (and the associated sequela) continues to be the bane of transplant immunology [2-4, 6, 15]. These complications are further magnified in the emerging field of VCA, as these grafts are not viewed as life-saving, presenting physicians with significant ethical dilemmas when identifying appropriate patients [1, 80]. For these reasons, it is imperative to develop tolerogenic strategies that are capable of reducing or eliminating the immunosuppression burden faced by VCA patients. Indeed, the future success and wider clinical application of VCA will not be realized until such tolerogenic strategies are implemented clinically.

The most commonly used immunosuppression drugs in the clinic today suppress allorecognition by targeting a single pathway [185, 186]. Given the complexity (and built-in redundancy) of the human immune system, it is not surprising that these primitive drugs possess limited effectiveness and present global side effects [33, 35]. In contrast, the mechanisms that our endogenous cells use to regulate these inflammatory processes to produce homeostasis in the healthy steady state are dramatically more sophisticated than what can be achieved by traditional immunosuppression [64, 65]. Indeed, evidence over the past ten years has implicated Tregs as playing a pivotal and well-documented role in resolving inflammation and promoting tolerance in transplant immunology [70, 71, 187].

In the wake of this evidence, there have been numerous clinical trials in which investigators utilized autologous *ex vivo* Treg cell therapy to treat a host of inflammatory pathologies including graft versus host disease (GVHD), type 1 diabetes and Crohn's Disease[76, 77, 168, 188]. Further, it has been shown in these clinical trials (and numerous other preclinical studies) that Treg cell therapy can be effective at ameliorating symptoms and is well tolerated in humans (from a toxicity standpoint)[75, 77, 168, 188]. However, the clinical implementation of *ex vivo* Treg cell therapy is nontrivial and presents a number of issues including the need for Good Manufacturing Product (GMP) facilities, expanding a sufficient number of Tregs *ex vivo*, addressing the propensity of Tregs to trans-differentiate into harmful effector T cells and the instability of FoxP3 as a transcription factor [70, 71, 81, 82, 168, 173, 189].

An alternative to *ex vivo* cell therapy that obviates some of the concerns previously mentioned is to enrich for endogenous Tregs *in situ*. One such approach utilized by our group and others has been to recruit circulating Tregs to a point source *in vivo* by establishing a functional gradient of the Treg-recruiting chemokine CCL22, which is a CCR4 agonist[131, 141, 142, 165, 166]. However, it is known that peripheral Tregs are particularly scarce, representing less than 2% of all Tregs in the human body[73]. 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 in more aggressive models of inflammation such as VCA. Therefore, 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.

In this study, we explored the potential of local Treg induction as a means to establish allograft tolerance in a stringent and clinically relevant model. To do this, we utilized a well-established rat hind-limb transplant model with Lewis rats serving as recipients and Brown Norway rats as donors. All animals receiving transplants were administered a short course of immunosuppression (consisting of three weeks of systemic (I.P.) FK-506 and lymphocyte depletion using ALS), to allow sufficient time for factors to diffuse from microparticles and influence the local microenvironment. Importantly, this also allowed us to demonstrate that TRI-MP could be used in tandem with commonly used immunosuppressive drugs.

Indeed, treatment with just two doses of TRI-MP at PODs 0 and 21 is able to significantly prolong rat hind-limb VCA survival when compared to control animals receiving just the baseline immunosuppression protocol (Figure 19B). Furthermore, to demonstrate that the combinations of all three factors in TRI-MP are responsible for the observed clinical result, individual microparticle formulations of TGF-β, Rapamycin, and IL-2 were also tested for their ability to prolong VCA survival. As shown in Figure 19B, none of the individual components could reliably confer the development of significant, long-term survival compared to recipients treated with TRI-MP. Treatment with Rapamycin MP was able to result in long-term survival in 3/8 grafts; this is not particularly surprising as Rapamycin is a clinically used immunosuppressive agent that has also been shown to induce Tregs [190]. While Rapamycin is not effective at preventing clinical VCA rejection in humans as a monotherapy, this result suggests that future Rapamycin-based controlled release systems (possibly used in tandem with FK-506) could potentially be modified to promote more reliable VCA survival. In addition to testing the individual components in TRI-MP, it was also demonstrated that each of the pairwise iterations of TRI-MP, were also not able to reliably prolong rodent VCA survival, suggesting that all three factors are necessary for Treg

induction and establishing graft tolerance (Figure 19C). This finding (along with results using the individual factors) is in line with other results published using TRI-MP *in vitro* and *in vivo* models of inflammation [128, 182, 183]. Finally, to illustrate the local effect of TRI-MP, we also treated a cohort of transplanted rats with TRI-MP as administered through injection in the contralateral (non-transplanted) limb (Figure 19C). While contralateral treatment with TRI-MP could not prolong long-term graft survival, rejecting grafts appeared to reject slightly later (10-14 days) than controls. Though this result is not statistically significant, it is certainly possible that Tregs were in fact induced in the contralateral limb, with their effectiveness being muted due to the fact that they were induced in the absence of antigen.

It was hypothesized that the primary mechanism by which TRI-MP induced VCA tolerance was due to the local induction of $FoxP3^+$ Tregs via naïve $CD4^+$ lymphocytes. This hypothesis appears to be consistent with the results of flow cytometric analysis of draining lymph nodes taken from TRI-MP treated grafts. Figure 22A demonstrates that there is a significantly higher percentage of $CD4^+CD25^{hi}FoxP3^+$ cells in the draining lymph nodes of TRI-MP treated grafts (normalized to naïve tissue) when compared to draining lymph nodes from rejecting. Because it is known that Tregs are able to inhibit the function of Teff phenotypes, we also examined draining lymph nodes for the presence of IFNγ, the characteristic Th1 cytokine. Indeed, we also observed a significantly lower percentage of $CD4^+IFN\gamma^+$ cells (normalized to naïve tissue) in the draining lymph nodes of TRI-MP treated grafts when compared to analogous tissue from rejecting grafts (Figure 22B). TRI-MP also appears to substantially reduce inflammation at the mRNA level as well. Expression of a panel of pro-inflammatory cytokines (TNF, Serglycin, Perforin-1, IL-17, IFNγ) are significantly increased in the draining lymph nodes of rejecting animals. However, data obtained from TRI-MP treated draining lymph nodes demonstrates that the expression of those

same genes is decreased. In fact, expression of these cytokines is comparable to that seen in naïve (untreated) lymph nodes (Figure 21).

To explore possible mechanisms for the clinical tolerance observed in Figure 19, the function and potential role of suppressive (Treg) and inflammatory (Teff) lymphocytes isolated from naïve and TRI-MP treated animals was assessed via a series of MLRs. As can be seen in Figure 23A, when comparing Treg depleted, $CD4^{+}CD25^{-}$ Teff populations from naïve and TRI-MP treated recipients, there is no significant difference in the ability of TRI-MP-treated Teff cells to respond to Brown Norway (donor) stimulation (note TRI-MP treated proliferation was normalized to Naïve proliferation), suggesting that $CD4^{+}CD25^{hi}$ Treg populations are likely contributing to the immune hypo-responsiveness toward BN antigen that was observed clinically. As shown in Figure 23B, Tregs isolated from TRI-MP treated recipients are significantly more effective at suppressing BN mediated Teff proliferation than Tregs isolated from naïve animals, suggesting that Tregs from TRI-MP treated animals may possess antigen specificity. To further investigate the role of antigen specificity, the ability of Tregs from TRI-MP treated recipients to suppress Teff proliferation in response to Brown Norway (donor) or Wistar Furth (third party) stimulation was tested. Indeed, Tregs from TRI-MP treated animals were superior at suppressing donor antigen-mediated Teff proliferation as opposed to third-party mediated suppression (Figure 24A), demonstrating Treg donor antigen specificity *in vitro*. Finally, we demonstrated that antigen-specific tolerance induced by TRI-MP was also applicable to *in vivo.* As can be seen in Figure 24B-C, TRI-MP treated animals with long-term surviving grafts (n=3) accepted secondary non-vascularized skin grafts from Brown Norway recipients, while rejecting grafts from Wistar Furth donors. Importantly, it should be stressed that animals that received secondary skin grafts had long-term surviving hind-limb allografts and had not received any systemic immunosuppression or

microparticle treatment for over 200 days, suggesting that the antigen-specific tolerance induced in these animals is long lasting and global.

In summary, the work presented herein demonstrate that a safe, cell-sized, dissolvable Treg Inducing microsphere formulation can prolong rodent hind-limb VCAs indefinitely in the absence of long-term, systemic immunosuppression. Furthermore, it was demonstrated that the prolongation of graft survival is due to the induction of antigen donor-specific tolerance. Finally, these results warrant future studies to examine the ability of TRI-MP to resolve inflammation and restore homeostasis in other pathologies characterized by aberrant inflammation including solid organ transplantation and certain autoimmune disorders.

7.0 CONCLUSIONS AND FUTURE WORK

The use of synthetic systems to influence the local cellular environment and enrich for specific cell populations is an attractive alternative approach to *ex vivo* cell therapy. Herein, we have described two systems that can locally enrich for suppressive Treg, resolve inflammation and promote dominant, antigen-specific tolerance in a rodent model of VCA. Notably, both of these formulations are able to achieve this in the absence of systemic, long-term immunosuppression.

In chapter 5, microspheres (referred to as Recruitment-MP) that are capable of establishing a physiological gradient of the Treg recruiting chemokine CCL22 were shown to prolong rat hindlimb allograft survival indefinitely. Specifically, it was shown that two subcutaneous intra-graft injections of Recruitment-MP administered on postoperative days 0 and 21 (in addition to a short course of immunosuppression, described in Figure 7) are sufficient for promoting VCA tolerance. Further, it was also shown that Tregs isolated from Recruitment-MP treated animals with long-term surviving grafts are more effective at suppressing donor (Brown Norway) stimulation than third-party stimulation in *ex vivo* proliferation assays. Finally, long-term surviving animals also displayed antigen-specific tolerance as evidenced by accepting non-vascularized skin grafts from Brown Norway recipients while rejecting third-party grafts.

Given the paucity of circulating Tregs, (less than 2% of all Tregs), recruitment of Treg populations could prove problematic when translating to preclinical and clinical studies. Thus, in chapter 6, an alternative system (referred to as TRI-MP) that is capable of releasing a combination of three factors (TGFβ, Rapamycin, and IL2) that are able to locally induce Tregs from naïve T cell populations was described. Indeed, TRI-MP was shown to promote indefinite VCA survival

(>300 days) when administered subcutaneously in the transplanted limb. Donor antigen-specific tolerance was also demonstrated using a secondary skin graft challenge.

The technologies described here are able to influence the immune system by providing key signals with the requisite temporospatial context, ultimately resulting in a restoration of homeostasis and (in the context of this thesis) antigen-specific tolerance toward a transplanted hind-limb. Given the promising results described herein, the immediate next step for both of these technologies is to test their ability to promote tolerance in a large animal model of VCA. Indeed, future studies are in place to test both Recruitment-MP and TRI-MP in a swine model of abdominal wall transplantation. In addition to VCA, it could potentially be transformative to the field of immunosuppression if these results could be translated to SOT as well. Future work developing alternative formulations and delivery systems for the factors described in this thesis will be imperative to allow for limited and minimally invasive delivery to a solid organ graft. Finally, it is also possible to envision strategies that utilize Treg induction and recruitment to resolve inflammation in autoimmune disorders and other pathologies characterized by disruptive inflammation.

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