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Nanoparticle use in Modulation of Transplant Rejection in a Murine Model

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Solid organ transplant has emerged over the last half century as an important treatment for solid organ failure. Management has matured dramatically over the past two decades with improvements in acute rejection, but long-term graft survival has improved very little and current treatment is limited by the side-effects and toxicities of immunosuppressive medications. Nanoparticle delivery of therapeutics, improving transport characteristics and decreasing systemic and local toxicity has emerged as a dynamic treatment modality, but little work has been done using nanoparticles in transplantation. Our research examined the use of CD4-targeted nanoparticles encapsulated with mycophenolic acid (MPA), a commonly used immunosuppressant in organ transplantation. This work is the first to examine antigen-specific targeting of nanoparticles in any transplant model. MPA-loaded particles show a slow and continuous release profile and biodistribution suggested retention in the spleen. Targeting of nanoparticles to CD4 T cells was suggested using *ex vivo* and *in vitro* flow cytometry. In the fully allogeneic MHCII mismatch BALB/C to C57BL/6 mice we found improved graft survival in the non-targeted MPA group and even greater graft survival in the CD4-targeted group. Targeted and non-targeted particle groups showed equal delay in rejection in the less immunogenic single MHC mismatch B6.H-2bm12 to C57BL/6 model that we showed to be CD4 dependent. In both models, graft survival times were increased over free drug and controls with roughly one thousand fold lower dose of drug in the nanoparticles as compared with free MPA. Consistent with these findings were decreased proliferation with targeted and non-targeted MPA-nanoparticles using *in vitro* and *ex vivo* mixed lymphocyte reactions. We postulated that the similar rejection times in targeted and non-targeted groups was due to dendritic cell (DC) involvement and we found active uptake of nanoparticles in DCs, a decrease in inflammatory cytokine production and a decrease in treated DCs ability to stimulate T cells via mixed lymphocyte reactions. Furthermore we found a possible mechanism in the DC interaction with T cells through the upregulation of the inhibiting co-stimulatory molecules B7-DC and B7-H1 on DCs treated with MPA-nanoparticles. We also found possible upregulation of CD4+CD25+ *Foxp3* expressing Tregs which may serve to increase graft acceptance. These results explore the involvement of dendritic cells in the process of nanoparticle-induced graft acceptance and suggest the feasibility of using nanoparticle drug vectors in clinical transplant.

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Introduction

Solid organ transplantation in humans has emerged over the past few decades as a major advance in life-saving treatments for a number of diseases (1), (2). Lung transplants treat incurable cystic fibrosis (3) and other pulmonary disorders, heart transplants, liver and kidney transplants treat a number of diseases that would eventually lead to patients death (4). Great advances have been made in the basic science understanding that we currently have of major and minor histocompatability, and the mechanisms of graft rejection. These advances have helped to improve both graft survival and long-term recipient survival (4). Medical treatment of transplant rejection has been primarily through immunosuppressive drug modalities to delay acute rejection. While usually successful at initially maintaining graft survival, long-term survival is often limited by patients' intolerance to the side-effects of chronic immunosuppression (5). Increased susceptibility to opportunistic infections limits chronic treatments. Additionally, immunosuppression leads to increased incidence of solid and hematologic neoplasms (6). Direct toxicity of these medications can lead to severe hypertension, diabetes, nephrotoxicity and many other severe complications and worsen the risk benefit ratio of long-term treatments. Therefore improvements in immune suppression after organ transplantation are clearly required.

With this in mind, the goal of treatment would be to reduce systemic toxicities and side-effects while maintaining therapeutic doses to the organ or cell population of interest. Traditional treatment modalities use systemic delivery and generally have narrow a therapeutic index. With variable bio-absorption and bio-availability, traditional systemic delivery of drug often falls outside of this narrow index with difficulty balancing

efficacy and toxicity. Moreover, the efficacy of systemic drug delivery is hampered by genetic polymorphisms (7), up-regulation of drug efflux pumps and development of resistance (8). With these problems as the driving force, nanoparticles have been utilized as a method of encapsulating toxic drug, allowing specific delivery and decreasing side-effects.

Background

Transplantation

In the 2000's there have been between 13,000 to 17,000 kidney transplants per year in the US (9). Liver transplants are the second most common with 5,000 to 7,000 per year. Heart transplants are third most common (1800-2300) and lung is fourth (1000-1500). Other solid organs such as pancreas and intestine, as well as combined transplants (heart/lung or kidney/pancreas) are less common (9). In 2008, only 6,000 of the 22,000 transplants were done with living donors, with the remaining from deceased donors, despite the dramatic difference in graft survival between living and deceased grafts (9).

The first successful solid organ transplantation was a kidney allograft in 1954 (4). By the end of the 1960's, liver, lung, heart and pancreas transplantation was operatively successful as well, despite extremely poor post-operative graft and host survival. In the mid 1970's cyclosporine was discovered by Jean Borel (10) and after approval in 1983 became a major breakthrough in the medical management of transplantation (11). Prior to this breakthrough, post-op survival times were dismal for patients receiving solid organ allografts. In the last twenty years our understanding of the immune system has allowed for human leukocyte antigen (HLA) typing and research has uncovered organ specific responses to transplant that have improved management. Organ specific metrics and measures by which to follow transplant function and rejection have been developed as well, and further drug development has created more immunosuppressive medications (4). These advances have continued to improve post-transplantation survival of patients. One study looked at changes in renal graft survival from 1988 to 1998. They found an improvement from 89.7% to 94.3% one-year-survival in living

donors, and an improvement from 76.0% to 89.3% one-year-survival in cadaveric grafts. These improvements occurred despite the use of older donors (which generally worsens graft function) in this study. There was also an overall decrease in acute rejection episodes which can be an important predictor for late rejection (12). Outcomes in other solid organ transplants such as heart and liver have similarly improved as medical management has evolved (4).

Transplant Rejection

There are generally three types of transplant rejection that are commonly seen: hyper-acute reactions immediately following reperfusion, acute rejection occurring days to years after surgery and chronic rejection that takes many years to manifest. Hyper-acute rejection occurs within minutes of the transplantation and is mediated by preformed antibodies against the tissue leading to rapid rejection. ABO blood type incompatibility is the most common cause of hyper-acute rejection, but it can also occur due to alloantibodies against major histocompatability complex (MHC) (Human leukocyte antigen or HLA is the human form of MHC). Preformed antibodies bind to the graft and the complement cascade is triggered, leading to complement-mediated destruction of the graft tissue (13). The blood clotting cascade is also initiated which leads to thrombosis and interruption of blood flow in the vascularized graft and tissue ischemia. In cases of hyper-acute rejection the graft must immediately be removed in order to prevent a systemic inflammatory response. This type of rejection is generally preventable by careful HLA and ABO matching and measurements of allograft-specific antibodies, but is not acutely treatable (13)

Acute rejection occurs from weeks to years after the transplant surgery. It is generally thought to be caused by HLA mismatch between graft and host. Even in carefully matched transplants, it is difficult to fully match all HLA types, and minor histo-incompatibility is likely present even in the best HLA matches. Acute rejection is common in all transplantation and does not usually lead to loss of the graft (14). A single episode if treated promptly is not a cause of concern; however, recurrent episodes have been shown to lead to increases in chronic rejection.

Acute rejection unlike hyper-acute rejection is a T cell dependent process. T cells can be activated through a direct and an indirect pathway. The indirect pathway occurs when host antigen presenting cells (APCs) such as dendritic cells, phagocytose foreign particles such as graft tissue. The host APCs migrate to the lymph nodes and spleen and present the foreign proteins via MHCII to T cells (Figure 1). The direct pathway occurs when resident donor APCs within the graft directly display foreign self protein via MHC to host T cells (Figure 1). In either pathway, the antigen specific T cell population is activated and T cell populations are increased that will target the graft tissue. CD8 cytotoxic T cells activated by MHCI and CD4 T cells activated by MHCII upregulate the inflammatory response through macrophage activation as well the humoral response. The direct pathway is thought to be more involved in acute rejection with direct T cell toxicity likely due to the higher frequency of directly allospecific T cells, but the indirect pathway is also involved in acute rejection. Indirect activation causes T cell activation, macrophage-induced tissue injury, fibrosis and alloantibody formation (15), (16) (13).

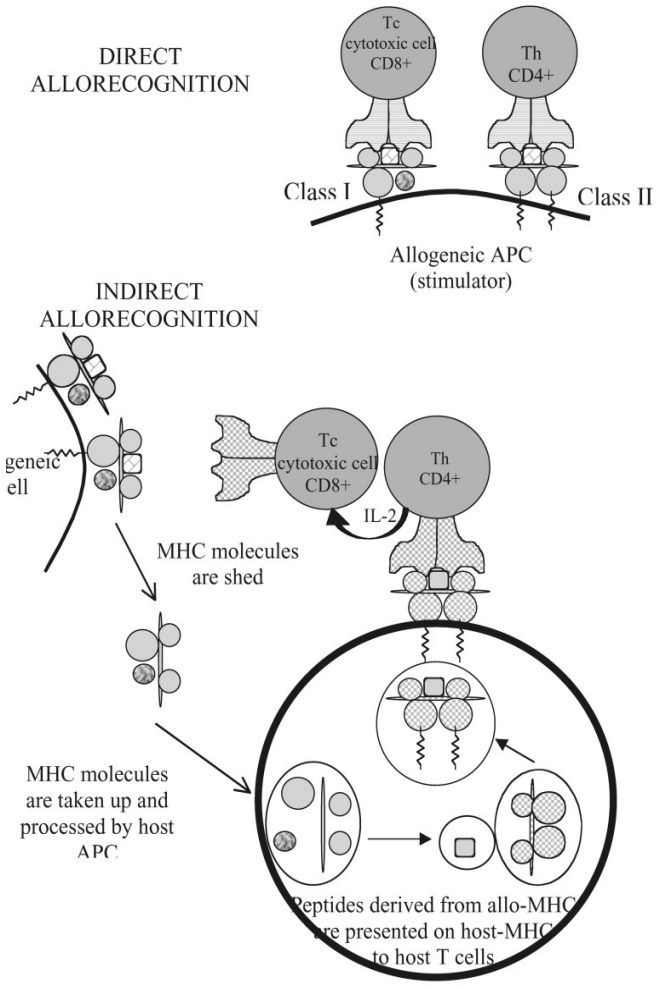

Figure 1. Schematic depicting methods of allorecognition in transplant rejection. **Direct Presentation**: Post transplantation, dendritic cells (DCs) from the donor leave the graft and circulate via lymphatics to regional lymph nodes and via blood vessels to the spleen. DCs present peptides from the donor on their own major histocompatibility complex (MHC) to the T-cells of the recipient, binding to the host T-cell receptor (TCR). Direct presentation brings about an alloreactive T-cell population that recognizes and kills the graft. **Indirect Presentation**: Post transplantation, host DCs migrate into the graft, and take up and process proteins from recipient cells. These host DC's migrate back to lymph nodes and spleen and present foreign peptides on self MHC, leading to an allo-specific response (15).

Chronic rejection occurs over very long periods of time leading to progressive vascular damage and insufficiency, concentric arteriosclerosis of graft vessels and parenchymal fibrosis and atrophy. Chronic rejection may be due to antigen-specific alloreactivity and antibody deposition, but may also be due to non-specific late effects of ischemia-reperfusion injury, long-term side-effects of cytotoxic drugs such as cyclosporine and even infection with cytomegalovirus. It has also been shown that the indirect pathway of allorecognition is involved with the pathogenesis of chronic rejection

as well (17). Unfortunately, chronic rejection is generally not amenable to treatment, and is the major cause of late graft dysfunction, occurring many years post-transplant. It is due to chronic rejection that despite improvements in short-term graft survival, long-term survival has seen little improvement, with a renal transplant half-life survival remaining at roughly 8 years (18), (5).

Medical Treatment of Rejection

Current medical management of transplant is aimed at modulation of acute rejection. Acute rejection is usually treated with short bursts of high-dose steroids which is often sufficient to prevent the rejection from progressing. Maintenance therapy is given to most patients which consists of chronic immunosuppression using multiple drugs in combination; often as “triple therapy”. Triple therapy includes a steroid with the addition of a calcineurin inhibitor such as cyclosporine or tacrolimus and an antiproliferative agent such as mycophenolate mofetil. Maintenance therapy serves to not only treat acute flares of rejection, but to prevent them from initially occurring. In some patients in which calcineurin inhibitors and steroids are contra-indicated, mTOR (mammalian target of rapamycin) inhibitors such as sirolimus may be substituted. In high risk patients, antibody treatments may be added as well to augment the response. The section below will briefly describe the types of medications currently available.

Corticosteroids: These medications are derivatives of the glucocorticoid family of steroids. The most commonly used is prednisone which is a synthetic analog of cortisol, and all drugs in this class share similarities in structure and function. They act via intracellular receptors that regulate transcription of specific genes. Up to 1% of genes

can be regulated by glucocorticoids, which cause a complex set of physiological effects. Their role in immune modulation lies in their ability to do the following: decrease IL-1, IL-3, IL-4, IL-5, IL-8, TNF-alpha and GM-CSF causing reduced inflammatory response, and reduced activation of the immune system. Decreased phospholipase A2 and cyclooxygenase also serve to decrease prostaglandins and leukotrienes (13). Steroids decrease adhesion molecules causing reduced emigration of WBC's out of blood vessels and increase the production of endonucleases causing increased apoptosis in lymphocytes. The negative side effects however are numerous, especially with long-term use. These include weight gain, diabetes, hypertension, fluid gain, electrolyte abnormalities, bone loss and skin thinning (19). The benefits therefore need to be carefully balanced against the side effects with long-term use.

Calcineurin Inhibitors: These medications block the calcium activated enzyme calcineurin, which is essential for the production of IL-2. T cell growth is mediated in large part by activation with IL-2, so calcineurin inhibitors serve to decrease T cell proliferation. They work very effectively to decrease the T cell response in acute rejection. These inhibitors also decrease IL-3, IL-4, GM-CSF and TNF-alpha, and inhibit B-cell proliferation (13). Cyclosporine and tacrolimus are the two drugs in this class that are commonly used in clinical transplant. Despite their more specific effects as compared to steroids, they can be very toxic as well especially to the kidneys and other organs. Tacrolimus is considered more powerful than cyclosporine and has a slightly better side-effect profile and is now used more frequently (20).

mTOR inhibitors: Instead of blocking the release of IL-2 as calcineurin inhibitors do, these medications bind to the mTOR complex thereby inhibiting the response to IL-2 and blocking proliferation of T and B cells. Sirolimus (also known as rapamycin) is the

major drug in this class. It is considered less toxic than the calcineurin inhibitors especially to the kidneys, and is generally well tolerated despite profibrotic and prodiabetogenic effects(20).

Antibody Treatments: These drugs are the latest in targeted therapies, and can specifically interfere with immune responses with less toxicity. Older versions like anti-lymphocyte globulin have been used for years as a non-specific treatment that can remove unwanted lymphocytes (they lack specificity however, and remove non-allospecific lymphocytes as well), but their use has been limited by side effects like serum sickness which is due to antigenicity after production in horses. However, more specific antibody treatments have developed as well. OKT3 is no longer commonly used due to a serious cytokine release syndrome and long term lymphoproliferative disorders, but was effective at specifically binding to T cells in the modulation of acute rejection. More recently, drugs like daclizumab have been created, employing a similar strategy to mTOR and calcineurin inhibitors by blocking IL-2, but instead via direct binding to its alpha subunit. The main advantage of these appears to be their reduction in opportunistic infection risk. (20) (13)

Antiproliferatives: These drugs are widely used in transplant and include mycophenolic acid, azathioprine and cyclophosphamide. They inhibit DNA synthesis and exert the majority of their pharmacologic action on rapidly dividing tissues. Azathioprine is converted within the body into a purine antagonist that competes with inosine monophosphatase inhibiting the synthesis of purines and decreasing DNA synthesis (13). Cyclophosphamide is an alkylating agent that directly incorporates itself into DNA . Both have numerous toxicities and side effects, with cyclophosphamide side-effects including hemorrhagic cystitis and bladder cancer (13).

Mycophenolic acid (MPA) has helped to dramatically decrease the incidence of acute rejection when used in combination with other medications. It has been approved for use in the prevention of acute rejection in renal, liver and heart allografts, and will likely soon be approved for use in pancreas and lung transplants as well (21). It is now being considered an excellent alternative to the older azathioprine, with better treatment of acute rejection. MPA blocks inosine-5'-monophosphate dehydrogenase (IMPDH), inhibiting the creation of guanosine. Lymphocytes lack the salvage pathway for creation of purines and are therefore stalled in their replication, with subsequent decreases in T cell proliferation and decreases in Ig production by B-cells (21). Furthermore, MPA has been shown to cause apoptosis in T cells (22), and affect other aspects of the immune system and inflammation as well. MPA has been shown to decrease the dendritic cell response, decrease maturation and prevent antigen presentation. It also has been shown to reduce monocyte proliferation and recruitment into sites of inflammation (21), and to decrease general inflammation through its actions on macrophages, decreasing nitric oxide and superoxide formation. MPA has also been shown to decrease arterial smooth muscle proliferation which can contribute to graft arteriopathy and rejection (22). It is used clinically in its pro-drug formulation as mycophenolate mofetil (MMF) which which is converted into MPA in the liver and was designed for use because of its superior bio-absorption. MPA has minimal side-effects compared with many other immunosuppressives which has made it an attractive medication. The side-effects however include infection risk, GI toxicities, thrombosis and rare severe complications (20).

Overview of Nanosystems

Nanotechnology was first discussed in 1974 by Norio Taniguchi in his paper “On the Basic Concept of Nano-technology”. He defined the technology based upon small particles in the nanometer range in size that behave as an individual unit in terms of their chemical and physical properties (23). As material technology has advanced, these particles have been created from numerous substances including metals, plastics, organic molecules, inorganic molecules like silica and a multitude of other substances. Due to this variability in design, the individual properties and functions of nanoparticles can be enormously different. Nanomedicine gained interest as an off-shoot of this technology for use in both diagnostic imaging as well as drug delivery, and over the past two decades there has been an explosion in research and development (24), (25), (26).

In particular, there has been considerable focus on using nanoparticles for drug delivery. Nanoparticles serve as an optimal drug delivery vector for a number of reasons. The creator has structural control over the size and shape of the drug cargo-space and the particles can serve as a precise scaffold or container with high drug carrying capacity; important in having plasticity in drug delivery. They can be made to be biocompatible with nontoxic polymers, allowing for non-immunogenic, non-toxic delivery within the body. They can have well-defined modifiable functionality for targeting with antibodies and ligands specific to any tissue or cell type within the body leading to decreased doses to tissues that may be sensitive to the drug, and increased doses to the tissues of interest. The nanoparticles can be created to undergo cellular adhesion, with subsequent endocytosis, and trafficking to allow delivery of drugs into the cytoplasm or nucleus. They can have well controlled and reproducible bioelimination or biodegradation, which is extremely important in understanding how to reproducibly

deliver drug for treatment. The nanoparticles can be developed to have controlled or triggerable drug release. Nanoparticles isolate and protect the encapsulated drug against inactivation during transit through the body to target cells. They can be designed with minimal nonspecific cellular and blood-protein binding properties in order to decrease non-specific uptake, decrease systemic doses and increase local doses. The nanoparticles can be created for easy and consistent reproducibility and synthesis. They can improve solubilization of the drug that may be non-soluble. They can be created for introduction via noninvasive routes. Ultimately these particles can have improved bioavailability and release profiles with reduction in administered drug doses, improving safety and diminishing side effects. Lastly, nanoparticles are an optimal drug delivery mechanism because they can be used as sustained release vectors, possibly improving compliance, quality of life and outcomes. Successful nanosystems also have the potential to evolve as we discover new proteins, receptors and ligands involved in diseases, offering opportunities to achieve drug targeting with newly discovered disease-specific targets (7).

Liposomal Particles

Liposomal particles are currently in use commercially for drug delivery of chemotherapeutics and antibiotics, and have long been studied for improving drug toxicities and delivery characteristics. These nanoparticles are composed of amphiphilic phospholipids that form bilayers with encapsulation of an aqueous interior. These small (80 to 100 nm) particles can be loaded with hydrophobic substances within the bilayer or hydrophilic substances within the aqueous core. Loading of drugs is achieved through a

variety of techniques that allow for encapsulation of drugs such as vincristine, doxirubicin and amphotericin (27) (28). The first generation of liposomal particles had the trouble of being rapidly cleared by the body due to rapid opsonization and uptake by the mononuclear phagocytic system. This issue was addressed with the use of poly-ethylene glycol (PEG) (8) coating which “hides” the particles from immune uptake and enhances distribution and bioavailablitity (29). PEGylated particles were found to localize in tissues with newly formed vasculature and in this localization a “passive targeting” (30) was found which underlies the utility in liposomal anti-cancer therapies causing relative increases in drug concentration within the leaky vasculature of tumors as compared with the normal vasculature of healthy tissues (31) (24). Furthermore, liposomal particles have the potential to be targeted via addition of antibodies onto their surfaces. There is much ongoing research looking at the utility of targeting drug encapsulated liposomal nanoparticles. However, liposomal systems in general have some disadvantages in terms of drug delivery. There is generally poor control over release of the encapsulated drug into the blood, causing unintended leak. There are also questions about the efficiency of encapsulation and the stability of the particles during longer storage(32)(33).

Dendrimer Particles

Dendrimer particles are polymerized branching structures emanating from a central core that roughly form a sphere in shape (34). The branching creates a labyrinthine core that can be utilized for entrapment of small substances. Furthermore, the terminal ends of the branches can be utilized and made functional via binding with

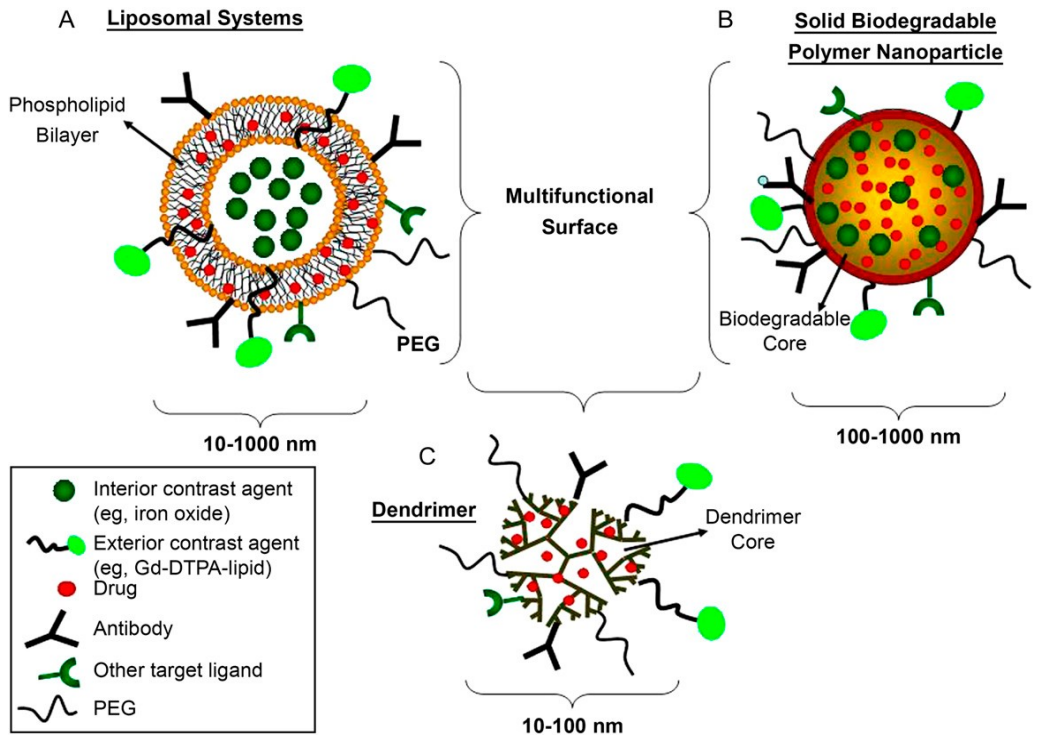

Figure 2. Aschematic created by our collaborators in the laboratory of Dr Fahmy. These represent the three more commonly used types of nanoparticles in medical practice. **A**. Liposomal systems are widely used in medicine with encapsulation of drugs such as doxirubicin and amphotericin. These particles consist of a lipid bilayer and an aqueous core. Hydrophobic drug can be carried within the phospholipd bilayer and hydrophilic drug can be carried in the core. **B**. These are solid biodegradeable polymer based nanoparticles such as those made with poly-lactic and glycolic acid (PLGA). These are flexible systems that allow for surface bound antibody targeting, central drug carrying and a controlled half-life. **C**. Dendrimer particles are finely branched structures with a central core. The branching creates a labrynth core for drug delivery and the ends of the branches can be functionalized with antibody for specific targeting.

ligand, antibodies, and imaging enhancing molecules. Our collaborators, Dr Fahmy and his lab have used these particles extensively, where they have utilized a polyamidoamine (PAMAM) dendrimer with PEG attached to the core for targeting T cell populations by coupling streptavidin to the terminal chain and using biotinylated antibodies against CD3 and peptide/MHC complexes. With doxirubicin bound to the

particles, their lab found excellent inhibition of polyclonal and antigen-specific T cells (32).

Solid Biodegradable Particles

Polymerized particles appear to have many advantages including biocompatability, biodegradability and functionalization with loading and targeting (35). Encapsulation using polymerized nanoparticles gives the operator specific and reproducible control of the biodegradability and release of the internalized drug (35). There are currently numerous materials used in the formulation of these types of nanoparticles, but particles made from polymers of lactic and glycolic acid (PLGA) will be focused on in more detail. These particles combine a hydrophobic poly-lactic acid (PLA) polymer with a hydrophilic poly-glycolic acid (PGA) polymer. These are desirable to utilize in a drug delivery system due to the fine control of the drug delivery that can be obtained by changing the relative ratios of each substance within the nanoparticle. PLA has a half-life of months and PGA of days, thereby giving the operator a range of options in terms of drug release by changing the PLA:PGA ratio (36). Drugs can be encapsulated and attached in a variety of ways including hydrophobic entrapment and direct conjugation to the PLGA surface, allowing a wide variety of substance to be brought together in one functionalized nanoparticle unit (37). Furthermore, this material has been shown to be safe in humans in thirty years of use (38) (39). The breakdown products of these particles, lactic and glycolic acid are physiologically normal substances involved in the Krebb cycle and are broken down into carbon dioxide and water without affecting normal cellular functions (40).

Micro-sized PLGA particles encapsulated with chemotherapeutic drugs have been shown to have improved drug efficacy compared with free drug (41). PLGA nanoparticles loaded with doxirubicin was shown to have prolonged release profile over a month and a similar effectiveness with *in vivo* injections in mice (42). Not only have PLGA particles been shown to improve drug delivery, but they have even been found in one paper to increase drug efficacy in cancer cell lines reversing multi-drug resistance (43). Currently, PLGA particles are found in clinical use as a depot form of GnRH called Lupron® (44).

Our collaborators in Dr Fahmy's lab have developed the ability to create nano-sized PLGA particles (Figure 3, 4), with encapsulation of both doxirubicin and mycophenolic acid, and direct conjugation of streptavidin onto the PLGA surface. With the addition of streptavidin, they have created a system that can attach any biotinylated antibody to the surface of the particle, theoretically allowing for targeting any moiety of interest that has a commercially manufactured biotinylated antibody (45) (32). They have also added PEG to the surface to improve circulation of the particles. Previously these particles were too difficult to manufacture and attach PEG. PEGylation has been shown to be very important in nanoparticle drug delivery by increasing transit times and decreasing non-specific uptake and binding throughout the body. The development of PEGylated solid biodegradable nanoparticles has lagged behind the development of liposomal PEGylated particles due to the technical difficulties in attachment to the surface and creation of functionalized particles (35). Our collaborators have successfully developed a technique that reliably and reproducibly functionalizes PLGA particle surfaces (Figure 3). They recently published a paper using PLGA encapsulation of doxirubicin where they were able to improve on the cytotoxicity while decreasing the

non-specific cardiotoxicity (35).

Dr Fahmy's lab has also been looking at targeting T cells for both imaging and drug delivery using these particles and antibodies against CD3 (46). Their lab suggested that due to their small size, the PLGA particles would be internalized into the T cells and would act as an intracellular drug reservoir allowing for sustained local release affecting only the cell population of interest. In their work they found the CD3 targeted particles encapsulated with doxirubicin were able to decrease proliferation of T cells after CD3 stimulation showing successful targeting and delivery of drug (32). These particles are also gaining attention by our collaborators as potential vehicles for vaccine delivery. The nanoparticles containing the viral proteins are internalized by both dendritic cells (DCs) and T cells which then become activated to prime a potent adaptive immune response (47)(48).

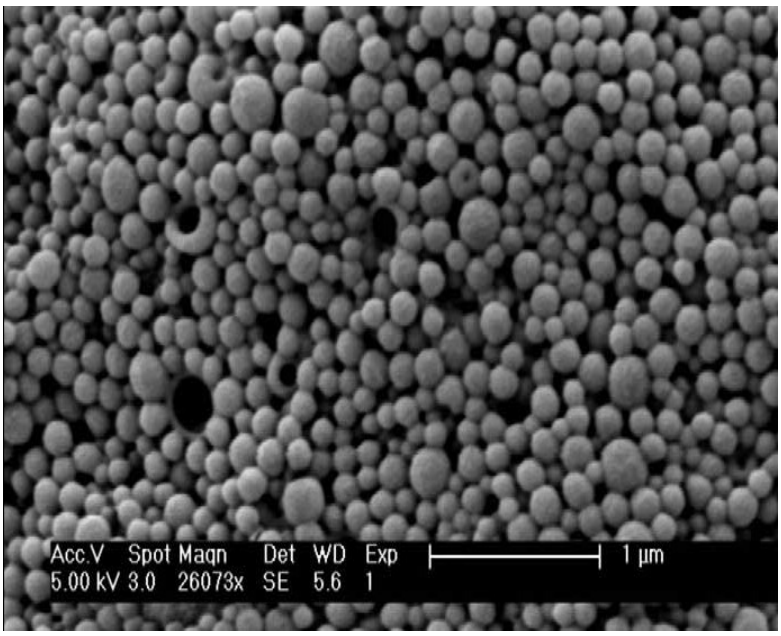

Figure 3. Electron Microscopy of [lactide-co-glycolide] (PLGA) particles. Particles were created in the lab of our collaborator Dr Fahmy. Production of PLGA nanoparticles results in small particles with an average size of 130nm. These particles can be encapsulated with a variety of substances including fluorescent imaging molecules such as rodamine and coumarin-6, drugs such as mycophelonic acid (MPA) and doxirubicin, and magnetic resonance enhancers such as gadalinium. The surface of the particles is used for attachment of avidin, and this can be utilized for the attachment of biotinylated antibodies.

Nanomedicine in Transplantation

The majority of research into nanoparticle drug delivery has examined uses in cancer treatment and diagnosis. The literature on nanoparticles in transplant rejection is far more limited. Very few studies were found in an extensive literature search on the use of nanoparticles in modulation of transplantation. One group has looked at PLA nanoparticles stabilized with cholesterol-modified chitosan encapsulated with rapamycin in a corneal transplantation rabbit model. This group found that the nanoparticles exhibited excellent retention in the precorneal area allowing for sustained release of rapamycin. When compared to topical free rapamycin they found significant efficacy in the treatment with similar (but slightly improved) mean survival times of the grafts (49). Two other groups looked at cyclosporine formulated into PLGA particles for use in corneal transplants. Both sets of researchers found significant increases in mean graft survival times in animals receiving the nanoparticles loaded with cyclosporine(50)(51)(52). Using PLGA encapsulated with tacrolimus, another group has also looked at corneal transplant rejection. Similarly to the groups using other immunosuppressives, PLGA loaded with tacrolimus delayed graft rejection and improved mean survival times (53).

Using liposomal encapsulations of tacrolimus and rapamycin, one group of researchers have published a number of manuscripts looking at modulation of transplant rejection in dopaminergic grafts in a Parkinson disease mouse model. These papers found decreased immunorejection of grafts in groups receiving treatment with liposomally encapsulated drug (54)(55)(56). Another group has been using liposomal preparations of tacrolimus in an islet cell transplantation model. They found significantly delayed graft rejection after using the liposomal formulation (57). This same Canadian

group also published a brief report indicating the improved side-effect profile and delayed skin and heart transplant rejection using oral liposomal tacrolimus (58). There have not been any skin or solid organ transplant models that utilize PLGA particles.

There is also a small body of research relevant for transplant that does not directly use a transplant model. One study of interest looked at nanoparticles and immune modulation, examining the role of PLGA particles loaded with rapamycin and their activity against dendritic cells. Interestingly as compared with free rapamycin which has little affect on dendritic cells, PLGA loaded drug caused a decrease in maturation markers such as MHC II, CD86 and CD40. In addition, PLGA delivered rapamycin decreased cytokine production and decreased T cell proliferation using mixed lymphocyte reactions (59). This same group has also used PLGA encapsulated rapamycin particles and looked at the expression of ICAM-1, an important adhesion molecule that facilitates the interaction of T cells with dendritic cells. They found that PLGA particles but not free-drug were able to down-regulate the expression of ICAM-1. Furthermore they found an immunosuppressive array of cytokine production with the rapamycin loaded PLGA particles (60).

Most other work in nanoparticles that is relevant to transplantation involve studies examining the encapsulation of immunosuppressive medications, but these studies do not directly look at their application in post-transplant treatment. One group successfully used PLGA encapsulated rapamycin (sirolimus) in a vascular re-stenosis model and were able to increase anti-proliferation with PLGA loaded drug as compared to free drug (61). Another group has looked at the use of oral PLGA particles loaded with cyclosporine. This study looked at pharmacokinetics and also found decreased renal toxicity in the oral nanoparticle drug vector as compared with the standard drug delivery

(40).

Research Question and Design

Our study examined the use of PLGA nanoparticles encapsulated with mycophenolic acid for the modulation of transplant rejection in a murine skin transplant model. We chose to use PLGA particles as these are considered by many (including our collaborators) to be the best system for sustained release, specific targeting, future clinical utility and can now be manufactured by our collaborators using what they consider a superior technique with the addition of PEGylation (35). We chose to use mycophenolic acid as our immune modulator as it is commonly used clinically and our collaborators have had success with its encapsulation within the PLGA particles. They have previously been working on using PLGA encapsulated with MPA in a lupus mouse model with preliminary results that have looked excellent (unpublished data). We chose to do skin transplants in mice as this is a commonly used model for transplantation that has relative technical ease. The skin transplants are highly immunogenic and their resistance to modulation makes them an excellent candidate for testing immune modulating treatments (16, 62). Furthermore, our lab has had great experience using this model in the past (63). As such, we looked at the use of MPA loaded particles, both targeted to CD4+ T cells and non-targeted, and compared the use of these particles to empty particles, free doses of MPA and non-treatment groups. We examined skin transplants in a major MHC mismatch BALB/C to C57BL/6 model as an initial test of MPA loaded particles to effect a response. We also wanted to test the ability to target specific cell populations using the targeted MPA particles. To accomplish this we used a

CD4-dependent transplant rejection model, using a less immunogenic single MHCII mismatch model with B6.H-2bm12 (spontaneous mutation of I-Ab region causing prompt rejection) to C57BL/6 mice. Our hypothesis was that specific targeted drug delivery via nanoparticles would increase graft acceptance and increase the mean survival times of transplanted skin in both our high and low immunogenic models. Our secondary aims were to analyze the role of cytokine signaling between treatment groups, analyze the expression of surface markers using flow cytometry and assess T cell proliferation with different treatment groups using mixed lymphocyte reactions. When our results showed that non-targeted nanoparticles also improved graft acceptance, we examined the impact of nanoparticle therapy on dendritic cells, particularly how particles impact costimulatory and coinhibitory pathways that DCs use to modulate T cell responses in rejection.

Experimental Methods

Animals

All animals used were housed in the Anlyan Center Animal Facility, and used according to Yale University IACUC protocol. BALB/C (H-2d), C57BL/6 (H-2b) and B6.H-2bm12 (H-2bm12) mice were purchased from the National Cancer Institute for our experiments and CD4 knockout mice from Jackson Labs. B6.H-2bm12 mice are a strain with a spontaneous mutation of the I-A^b molecule resulting in a 3-aa substitution in the hypervariable region of the Aβ chain. They are otherwise identical to C57BL/6 mice. All animals were housed under pathogen-free conditions, and animals were not used if they had any skin lesions or other signs of sickness.

Nanoparticles

All nanoparticles were created in the lab of Tarek Fahmy, by our collaborator Michael Look, a PhD candidate in Bioengineering. Nanoparticles were made of polymers of lactic and glycolic acid, with avidin impregnated into the surface. 20 mg of dry MPA was added in 400 uL of methanol (50 mg/mL stock) and subsequently added to 2 ml ethyl acetate containing 200 mg polymer and the methanol was evaporated off. Dropwise, 2mL of organic phase of MPA/PLGA/ethyl acetate was added to 4 mL of a 3.75% PVA solution containing 5 mg palmitate-avidin under vortex. The solution was sonicated under ice at 38% amplitude, 3X for 10 seconds to form an emulsion. Nanoparticles were added to a beaker of 100 mL of 0.3% PVA and stirred in the dark for 3 hours at room temperature. After 3 hours of incubation, particles were collected into

centrifuge tubes and spun at 12000 rpm for 12 minutes at 4ºC. Supernatant was dumped and particles thoroughly resuspended in ~40-50 mL d.i. water using a sonicator. Particles were spun and washed once. Particles were collected in a single 50 mL falcon tube in ~5-10 mL d.i. water. Particles were passed through a 40 um filter to remove aggregates (and PVA debris), and frozen at -80°C for at least 1 hour. Particles were lyophilized for 48 hours in the dark. The nanoparticle yield from this protocol had a theoretical maximum avidin coverage of 25 ug avidin-palmitate per mg of polymer. Coumarin-6, and rhodamine particles were created similarly but with loading of dye instead of drug (Figure 4) for fluorescent imaging.

In preparation for injection into mice for treatment of transplant rejection, nanoparticles were prepared for targeting or non-targeted groups. All nanoparticle preparation prior to injection was done by either Dr. Anushree Shirali, (nephrology fellow), or Elias Kassis. In the targeted groups biotinylated RM4-4, a monoclonal non-depleting antibody binding CD4 was attached to the streptavidin moieties of the nanoparticles. Nanoparticles were weighed, suspended in PBS and incubated with RM4-4 antibody at a concentration of 7.5ul (0.5mg/ml) biotin per 1mg particle. PEG was added at 1:1 molar ration(M.W. 10000) at 0.75 ul (5mg/ml). After 30 minutes at room temperature, particles were spun down, the supernatant removed and the particles re-suspended in PBS to obtain an injection dose of 20mg/ml (250 ml injection: 5mg/animal). The non-targeted particles were incubated with PEG alone. MPA-loaded, empty, coumarin-6 and rhodamine particles were prepared identically for drug delivery and fluorescent imaging.

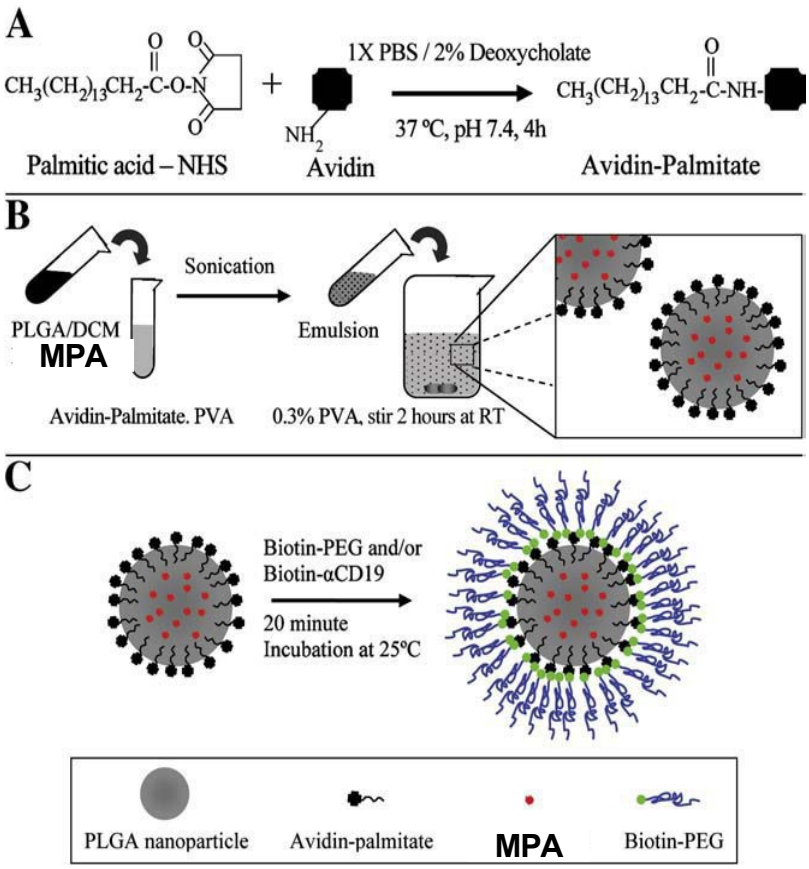

Figure 4. Figure taken from our collaborators (35), showing a schematic of PLGA nanoparticle creation. A) Synthesis of avidin-lipid conjugate. Avidin is reacted with palmitic acid–NHS in a 2% deoxycholate buffer to form avidin-palmitate conjugate. (B) The conjugate is then added during the aqueous phase of a modified single-emulsion technique to form avidin-coated PLGA nanoparticles (C) Avidin-coated nanoparticles can then be incubated in solution with biotinylated ligands to form surface-modified nanoparticles.

MPA Release From Particles

This experiment was done by Michael Look in the lab of Dr Tarek Fahmy. 1-10 mg/ml of MPA-loaded particles were prepared in PBS. The particles were place in a microcentrifuge tube and placed on a shaker at 37ºC. Once per day the tubes were spun at 13200 rcf for 10 minutes. The supernatant was collected and stored at -20ºC until ready for use. The pellet was resuspended in 1ml PBS and allowed to incubate for 24 hours until the next supernatant collection. This continued for one week. The supernatants were analyzed for MPA on a SpectraMax M5 plate reader (Molecular Devices) with excitation 340nm and emission 450nm with these data points plotted to

determine MPA release.

Transplantation

All transplantation was performed by either Elias Kassis (BALB/C) or Dr Anushree Shirali (B6.H-2bm12 mice). On the day of transplantation, donor mice were shaved, cleaned with alcohol and iodine and sacrificed using isofluorane, and skin was removed. Fascia was cleared from under the dermis and skin was placed in PBS on ice until ready for transplant. Recipient mice were anesthetized with isofluorane shaved, cleaned with alcohol and betadine. With fine scalpels a 2x2 cm square area of skin was removed from the right dorsal thorax. The donor skin was cut to size and attached to the host using skin staples. Bacitracin was placed topically and a bandage put in place. The bandages were removed at 1 week post-operatively. Mice were given carprofen in their water for analgesia as per standard protocol.

At day 0, day 4, day 7, day 14 and 21, animals were injected with their treatments (Figure 5). Treatment groups were; **Group 1**: Control, PBS treatment. **Group 2**: Non-targeted empty particles at 5mg per animal per treatment time. **Group 3**: free mycophenolic acid at 5mg total dose injection per animal per treatment time (initially intermittently at day 0, 4, 7, 14, 21. In a separate group [n=3] free MPA was given daily). **Group 4**: Non-targeted nanoparticles loaded with mycophenolic acid at 1-10ug/mg particles, given in total injections of 5mg particles per animal per treatment time. **Group 5**: CD-4 targeted (RM4-4 antibody) nanoparticles loaded with mycophenolic acid at 1-10ug/mg particles, given in total injections of 5mg particles per animal per treatment time (Figure 5). Injections were done intraperitoneally. Animals were followed for rejection

with complete rejection the primary end point. Complete rejection was considered to be >70% scabbing over of the graft, or shedding of the graft. In transplanted animals treated with GK1.5, animals were given 150 ug intraperitoneal injections at five times points (day 0, 2, 10, 14, 21). Transplants done with CD4 knockout animals were done identically as the other transplanted animals but no treatment was given and animals were followed for rejection.

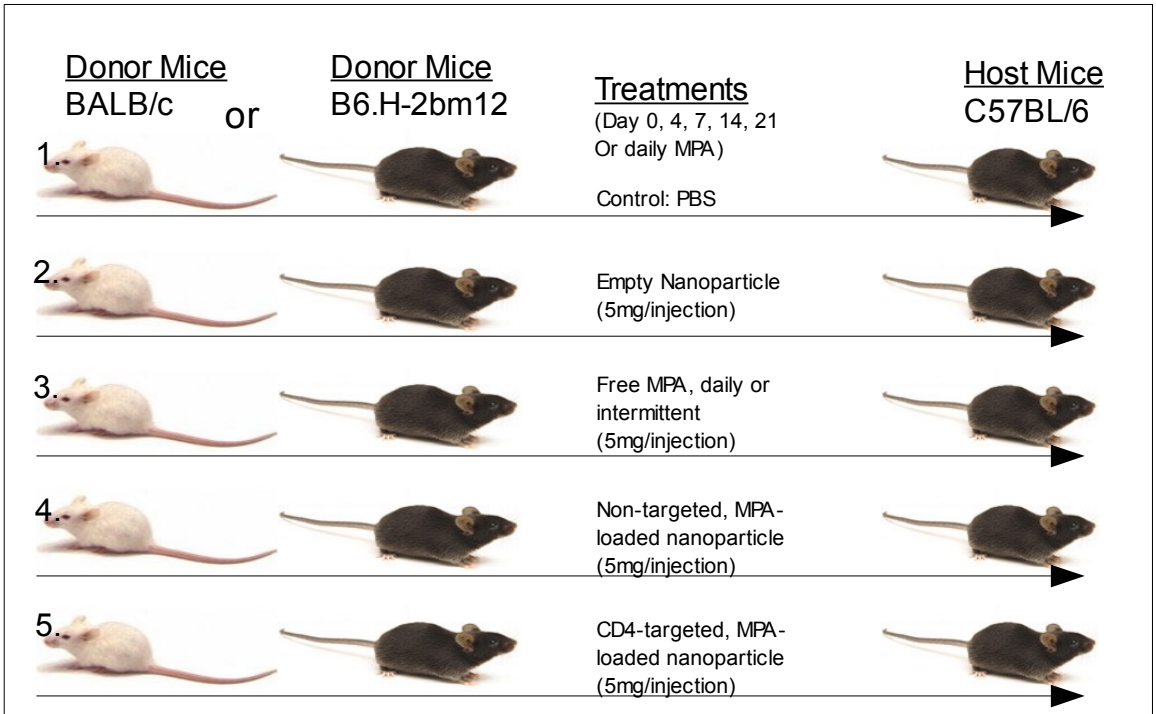

Figure 5. Study design for transplantation. BALB/c or B6.H-2bm12 mice are used to harvest donor skin and C57BL/6 mice are used for the host. Host mice are treated on day 0, 4, 7, 14, 21 with treatment groups. After skin transplantation, mice are measured for graft survival, used to examine *ex vivo* proliferation of T-cells via mixed lymphocyte reaction, dendritic cells collected from draining lymph nodes and measured for maturation markers, numbers of regulatory T-cells examined in peripheral blood, and graft infiltration determined by immunohistochemistry after biopsy of the graft.

Dendritic Cell Collection

Dendritic cells were collected and grown by both Elias Kassis and Dr. Anushree Shirali. Dendritic cells were harvested from post-treatment mice (transplant mice after

complete rejection, or at specific time points), as well as naive mice that had not received a skin transplant. Long bones of the mice were crushed using a mortar and pestle and bone marrow was filtered from tissue and bone while rinsing with PBS. RBC's were lysed using a hypotonic ammonium chloride buffer. Mixed bone marrow cells were purified for dendritic cells using complement binding and killing with antibodies against GK1.5, B220, GK2.43, TIB211, TIB120 and rabbit complement incubated for one hour (Gift from the lab of Dr Ira Mellman). After purification and counting using a hemocytometer with 64x64 grid, dendritic cells were plated in 24 well plates at 1million cells/well and allowed to grow for 3-5 days in the presence of 1% GM-CSF in RPMI media at 37º C in a 5% CO2 incubator.

Dendritic cells used for MLR or cytokine analysis. On day 3 or 5 post-incubation, wells of dendritic cells were treated with MPA-nanoparticles at 1mg/ml, 0.1mg/ml and 0.01mg/ml. Empty nanoparticles were given at the same dosing. Free MPA was given at doses of 5-10ug/ml, 0.5-1.0ug/ml, 0.05-0.1ug/ml and 0.005-0.01ug/ml. The range in dose was due to different batches of nanoparticles and slight difference in loading of MPA in each batch. The free MPA was given to roughly match the total amount of MPA per MPA-loaded nanoparticle dose. On day 5 or 6, some dendritic cells were stimulated with LPS (25-50ng/ml) as a maturation stimulus, and allowed to incubate for and additional 24 hours before use.

Splenocyte and CD4 Collection

Spleens were collected from mice, homogenized, and filtered through a mesh screen by either Elias Kassis or Dr Anushree Shirali. RBC's were destroyed using RBC

lysis buffer (hypotonic ammonium chloride buffer) and cells were either set aside for use or enriched for CD4 cells. CD4+ cells were enriched via negative selection using a kit from EasySep according to the manufactures instructions (Stecell Vancouver, Canada) that negatively selected for CD4 cells using magnetic particles and removing CD8+, CD11b+, CD19+, CD45r+, CD49b+, Ter119+ cells. Enriched CD4 T cells were then used for MLR.

Mixed Lymphocyte Reactions

Mixed Lymphocyte Reactions (MLR's) were undertaken using purified dendritic cells or splenocytes from the donor BALB/C or B6.H-2bm12 mice cultured with CD4 enriched splenocytes from C57BL/6 host mice and were done by Elias Kassis and Dr Anushree Shirali. In the initial BALB/C to C57BL/6 *ex vivo* experiment, mixed BALB/C splenocytes (at $1x10^6$ cells/well) were incubated with C57BL/6 CD4 cells that had been isolated and pooled from mice on day 35 post-transplant (at 1×10^5 cells/well). Cells were plated at 1:10 ratio (CD4:DCs) per well and allowed to co-incubate for 48-72hours. In the *in vitro* studies, dendritic cells were incubated with nanoparticles encapsulated with MPA (at 1mg/ml, 0.1mg/ml, 0.01mg/ml and 0.001mg/ml depending on the experiment), empty nanoparticles (at the same doses as loaded particles), free MPA (at 8-10ug/ml, 0.8-1ug/ml, 0.08-0.1ug/ml and 0.008-0.01ug/ml), and no treatments and then stimulated with LPS (as described in the dendritic cell section) prior to coculture with the C57BL/6 CD4 cells. In all experiments, the cells were co-cultured in Bruffs media containing 10% FCS, 1% pen/strep, 50um 2ME and 2 mM L glutamine. ^{3}H-thymidine was then added and the cells incubated for an additional 18-24 hours at 37º C in 5%

CO2 incubator. Cell proliferation was measured using a *Beta* plate scintillation counter after washing and extracting ^{3}H-thymidine from the proliferative cells using a cell harvester from Scatron Instruments.

ELISA

ELISA analysis was done by either Elias Kassis or Dr Anushree Shirali. Mixed lymphocyte reactions (as described) had aliquots of the supernatants collected for analysis. DCs treated with MPA encapsulated nanoparticles, empty particles, free MPA and no treatment were stimulated with LPS (50ng/ml) and after 24 hours the cells were spun, and the supernatant collected for analysis. 96 well plates from Costar were used for culture. ELISA kits from *eBioscience* were used. Capture antibody (IL-2, IL-6, IL-10, IL-12, TNF, IFN) was attached overnight at 4ºC at 1:250 dilution in ELISA buffer (*eBioscience* ELISA capture buffer). Plates were washed 5x and were blocked for one hour at room temperature with ELISA assay diluent (*eBioscience* assay buffer provided at 5x solution). After blocking supernatant samples were plated along with the standard curve dilutions. The samples were incubated overnight at 4ºC and after washing were incubated with detection antibody at 1:250 dilution in ELISA assay diluent with avidin-HRP for an additional 30 minutes and finally with superaqua blue solution (*BD bioscience*)until full development. Standard curves were created in duplicate using standards for each antibody from *E-bioscience.* The plates were read with a Synergy HT well reader at an absorbance of 450nm.

Flow Cytometry

Flow cytometry was done by Elias Kassis and Dr. Anushree Shirali. Cells were collected from transplant treatment groups *in vivo* by harvesting the spleen or draining lymph nodes of transplanted mice. Cells were also collected from *in-vitro* cell stimulations as described in early sections. Fluorescent antibodies against CD4, CD3, CD11c, CD25, CD40, CD80, CD86, MHCII, B7-DC, B7-H1, Thy1.2, Annexin, 7-AAD, *Foxp3* were purchased from *eBioscience* and *BD Pharmingen.* These antibodies were incubated with the cells at their appropriate dilution for 30 minutes at 4º and spun twice to remove excess antibody with resuspension in PBS. Cells that were not immediately analyzed were fixed in formalin-based fixative (*BD cyotofix/cytoperm).* Cells stained with *Foxp3* were permeabilized and fixed prior to incubation with antibodies as *Foxp3* is an intracellular transcription factor. The cells were analyzed using the FACS CALIBUR flow cytometry machines in the Yale Anlyan Center flow cytometry center. Cells were gated on lymphocytes and dendritic cells using a side scatter and forward scatter setting that had been previously established by a member of our lab. Single stained controls were used to set the gain and voltage in each channel and prevent overflow into neighboring channels and the final data were uploaded and analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Histology, and Fluorescent Microscopy

Biopsies of the grafts were obtained at 15 days post-op from representative mice by Dr Anushree Shirali. The biopsies were formalin fixed (10% formalin), and paraffin mounted on glass slides. The tissues were H&E stained and then examined for cellular

infiltration, signs of inflammation and other tissue characteristics using a Leica DM IRB inverted microscope.

Dendritic cells were harvested and cultured for five days as previously described. On day 5, $1x10^6$ cells were collected and plated on lab-tek chamber slides (Nunc) over-night for attachment by Elias Kassis and Dr Anushree Shirali. Coumarin 6 loaded nanoparticles and empty nanoparticles were incubated with the plated dendritic cells. The slides were either incubated for 2 hours or 18 hours, then washed 3x with cold PBS. 4% paraformaldehyde was used to fix the cells on ice for 15 minutes. Cells were again washed were 3x with cold PBS, excess PBS was gently aspirated, and 2 drops of Anti-Fade Gold w/DapI was added. A coverslip was placed over the cells prior to examination under fluorescent microscopy using the green channel of the Leica DM IRB microscope.

Nanoparticle Biodistribution

To determine the biodistribution of targeted and non-targeted nanoparticles, Noah Capurso an MD candidate in Dr Tarek Fahmy's lab used rhodamine encapsulated nanoparticles with either PEG alone or CD4/PEG bound to the particles as was previously described in the nanoparticle methodology. Particles were injected retro-orbitally into one mouse/group/time point of harvest. Targeted particle animals were sacrificed at 1, 5 and 24 hours and non-targeted particles were harvested at 3, 8 and 24 hours. In our laboratory, Elias Kassis and Dr Anushree Shirali compared CD4-targeted and non-targeted coumarin-6 loaded particles with empty particles. As a control, three naive C57BL/6 mice were given intraperitoneal injections of empty non-targeted

nanoparticles at a total dose of 5mg. Non-targeted coumarin-6 loaded nanoparticles were injected into a second group of animals, and targeted coumarin-6 loaded particles injected into a third both at 5mg total dose as well. After three hours, the mice were sacrificed. Serum was collected by removal of blood via cardiac puncture, the blood allowed to clot, spun and serum removed from the remaining clot. The liver, spleen, kidneys, lungs and heart were all removed, weighed and homogenized in 1 ml of deionized water. The tissues were frozen overnight at -80ºC and after thawing were mixed at 1:2 volume with 1% Triton X-100 in DMSO, incubated for 1hour at 37ºC to extract dye and Frozen again overnight at -80ºC. Homogenate was spun the following day at max rpm for 10minutes and 200ul of the supernatant was transferred onto a black 96well ELISA plate for fluorescent reading. Standard curves were created using serial decreasing concentrations of known coumarin-6 concentrations in the extraction solution. Standards and samples were read using a fluorescent plate reader. The total mass of coumarin-6/mass of tissue was calculated using the weights of the removed organs.

Statistical Analysis

Comparison of means was performed using two tailed T-tests, and repeated measures were compared using analysis of variance (ANOVA), with Bonferronis post-test comparison of means. Survival data between groups were compared using a Log-Rank analysis. All results were evaluated using Graphpad Prism statistical software (San Diego, CA, USA). Statistical significance was considered by a p value of <0.05. Results are reported with mean values ± standard error.

Results

Nanoparticle drug kinetics and distribution

With the design of clinically used medications, it is important to develop drugs that are predictable regarding their release and distribution. To understand and evaluate the drug release from the particles, our collaborators looked at MPA-loaded nanoparticles in solution (at physiological pH and temperature designed to simulate *in vivo* conditions) and measured the release of MPA within solution over the course of a week. They showed the constant release profile of the nanoparticles with a linear release rate. The release of MPA from the nanoparticles into the surrounding solution remained steady throughout the incubation with slow and sustained release over a seven day period (Figure 6).

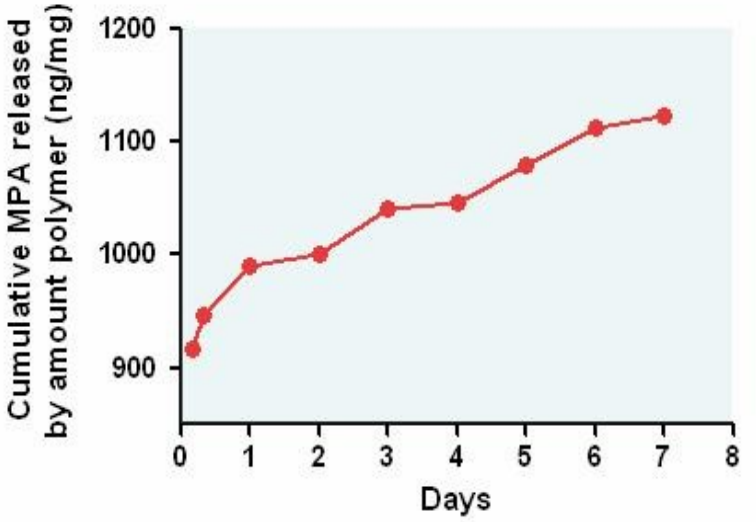

Figure 6. Controlled release graph of cumulative MPA release from nanoparticles loaded with 1 µg MPA/mg particle in phosphate buffered saline (PBS) buffer at 37 °C, pH 7.4. The assay shows that after an initial steep rate of release of encapsulate MPA, there is a constant rate of release.

To characterize the distribution of nanoparticles within our murine model our collaborators did a biodistribution assay of rhodamine-loaded particles. They looked at total fluorescence of each organ (1,5 and 24 hours for CD4 targeted and 3,8 and 24

hours for non-targeted) after retro-orbital injection of CD4-targeted and non-targeted particles. Fluorescence was converted to ng rhodamine per gram of organ using a standard curve and organ weights, and total rhodamine compared between groups. They found increased levels of rhodamine in the spleen (68 ng rhodamine/g organ) at 24 hours, in the CD4 targeted group and only 4.8ng rhodamine/g organ in the non-targeted group, but only did the study in one animal per group (Figure 7). Overall, the measured levels of nanoparticles were much higher in each organ in the CD4-targeted group, but only the 24 hour time point is directly comparable between groups for each organ due to the differences in other time points.

We also looked at the biodistribution of the nanoparticles within the mouse after intraperitoneal injection, comparing CD4 targeted-coumarin-6 loaded particle, non-targeted coumarin-6 loaded particles loaded, and empty nanoparticles. We harvested the organs three hours post injection, but did not see any difference in fluorescence between targeted and non-targeted treatments (data not shown).

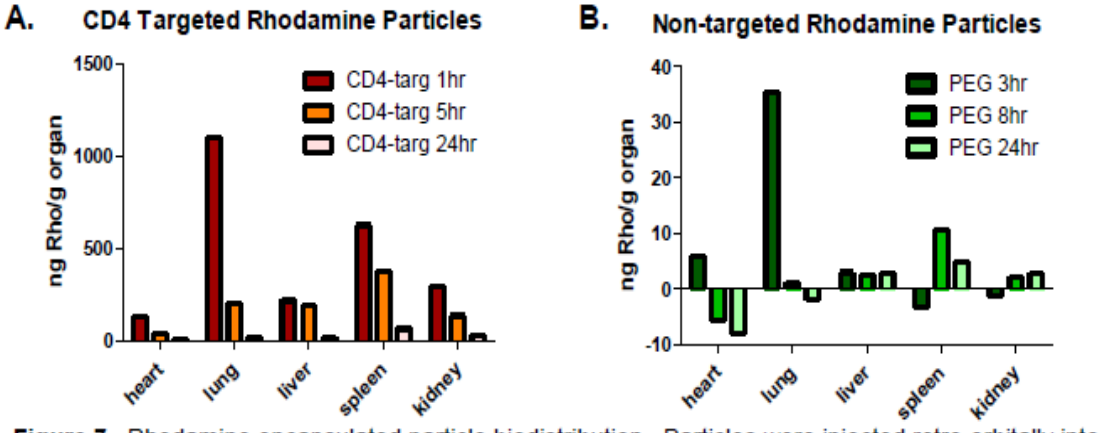

Figure 7. Rhodamine encapsulated particle biodistribution. Particles were injected retro-orbitally into mice and organs were harvested at the indicated time points with each organ processed to extract fluorescence (n=1mouse/time point/organ) **A**. CD4-targeted particles showed increased retention in the spleen. **B**. Non-targeted PEGylated particles showed initially high levels in the lungs similarly to CD4 targeted particles, but had little retention at later time points.

CD4 Targeting Flow Cytometry

Since we were using nanoparticles with CD4 targeting through the RM4-4 antibody, we wanted to determine if specific targeting was actually occurring in our system. We examined *in vitro* CD4 targeting using flow cytometry. Splenocytes were cultured in seven groups (n=1/group) with staining using CD3-PE in the FL2 channel, CD4-PerCP-Cy5 in the FL3 channel and CD11c-APC in the FL4 channel. Treatment groups were the following: no treatment, high (0.1mg/ml) and low (0.01mg/ml) concentrations each of CD4-targeted particles without coumarin-6, non-targeted isotype control (to RM4-4) coumarin-6 loaded particles, and CD4-targeted coumarin-6 loaded particles. The results are reported as high and low concentrations of each particle group (Figure 8,9). The targeted coumarin-6 particles showed greater mean fluorescent intensity in both high (MFI = 157) and low concentrations (MFI = 52) when compared to equivalent concentrations of non-targeted coumarin-6 loaded particles (high concentration MFI = 62, low concentration MFI = 27), suggesting the effectiveness for targeting CD4 cells with nanoparticles (Figure 8). When the CD3+CD4+ cell population was gated upon similar trends were revealed with increased mean FL1 fluorescence in the CD4 targeted groups (high concentration MFI = 636, low concentration MFI = 176) vs non-targeted groups (high MFI = 83, low MFI = 24) (Figure 9).

The experiment was repeated in an *ex vivo* model in four groups (n = 3 per group) using a single dose (5mg of injected particle) of each particle group instead of a high and low dose (as in the *in vitro*). Animals had their spleens harvested four hours after the injection of the treatments. Mixed splenocytes were stained using CD11c-PE in the FL2 channel and CD4-PerCP-Cy5 in the FL3 channel. The trends were maintained in this experiment as compared with the *in vitro* data, with increased mean FL1

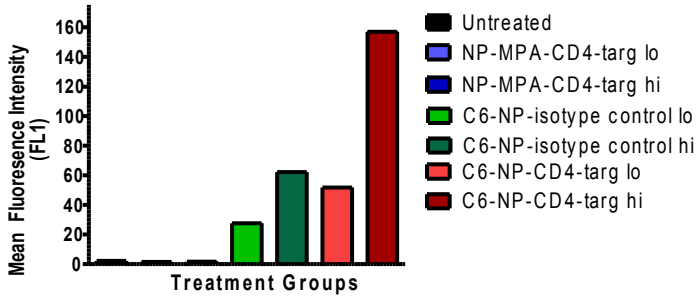

Figure 8. Bar graphs showing comparisons between FL1 channel expression (coumarin-6) between low and high concentrations (0.1mg/ml and 0.01mg/ml) of CD4-targeted and non-targeted nanoparticles. Isotype control was used as comparison to RM4-4 CD4 targeting antibodies. Splenocytes were collected from naïve mice, were cultured *in vitro* with treatment groups and stained with CD3-PE in FL2, CD4-PerCP-PECy5 in FL3 and CD11c-APC in FL4. N=1 per group

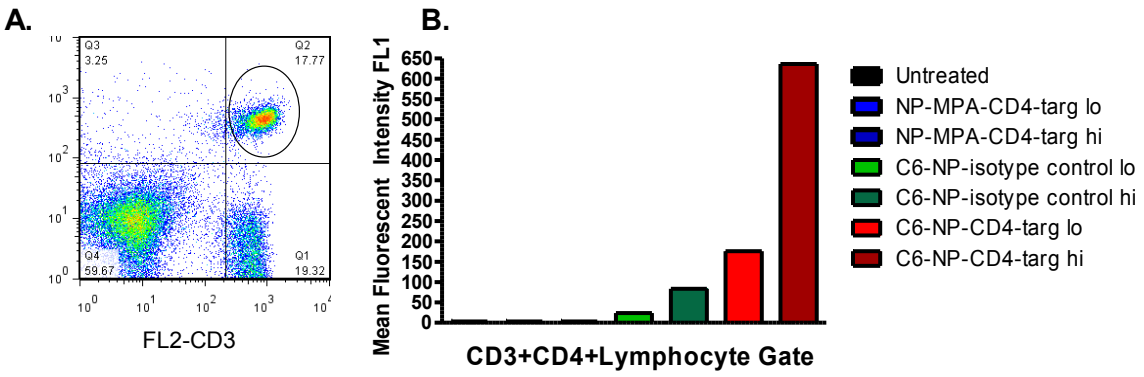

Figure 9. **A**. CD3 (FL2 channel) and CD4 (FL3 channel) gating showing a distinct CD3+CD4+ double positive population in the untreated control. **B**. Comparison of FL1 channel expression (coumarin-6) in the CD3+CD4+ double positive population between hi and low concentrations (0.1mg/ml and 0.01mg/ml) of CD4-targeted, non-targeted nanoparticles loaded with coumarin-6 and CD4-targted particles not loaded with coumarin-6. Splenocytes were cultured *in vitro* with treatment groups and stained with CD3-PE in FL2, CD4-PerCP-PECy5 in FL3 and CD11c-APC in FL4. N=1 per group

fluorescence in the CD4-targeted groups (MFI = 26.8±10) as compared to the control (MFI = 3.5±0.75) (Figure 10). However, despite much greater mean fluorescence in the CD4 targeted group, it was approaching statistical significance (p=0.08), but was found to be similar to both the isotype control-coumarin-6 loaded (MFI = 10.9±1.6) and the control, likely due to one low outlier in the CD4 targeted group (Figure 10). In these same splenocytes stained for CD11c (in the FL2 channel) and CD4 (in the FL3 channel),

it was found that a higher percentage of FL1 (coumarin-6) fluorescent cells are CD4+CD11c- in the CD4-targeted group (17% in CD4-targeted vs 10% in the non-targeted group)(Figure 11, Quadrant 1). In the non-targeted group, a higher percentage of FL1 fluorescent cells were CD11c+CD4- (9.4% in the non-targeted vs 4.6% in the CD4-targeted) (Figure 11, Quadrant 3).

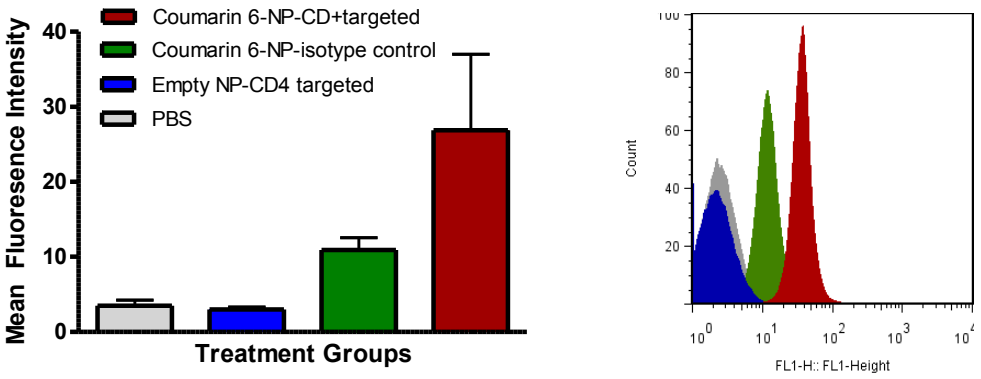

Figure 10. *Ex vivo* study looking at CD4 targeting in nanoparticles after collection of splenocytes from mice 4 hours post injection with treatment groups and stained with or CD11c-PE (FL2 channel), CD4-PerCP-Cy5 (FL3 channel and Thy1.2-APC (FL4 channel). FL1 measures coumarin-6 in nanoparticles. Animals were injected with PBS, empty CD4-targeted nanoparticles (5mg particles), coumarin-6 loaded particles (5mg particles) with isotype control or CD4 targeted coumarin-6 loaded particles (n=3 for each group). There was more fluorescence in the CD4-targeted particles than in the control and isotype controls, but due to one outlier, the increases in fluorescence were not statistically significant despite a large difference in mean fluorescence intensity.

BALB/C to C57BL/6 grafts

To determine the effectiveness of the MPA-loaded nanoparticles in modifying the transplant rejection response, we first examined a full MHC mismatch BALB/C to C57BL/6 skin transplantation (Figure 12). As expected, rejection overall was rapid in our controls, but in this system we found significantly delayed graft rejection in non-specific nanoparticles (NP-MPA, 1ug MPA loaded per mg NP, 5 mg NP-MPA dosed per mouse on day 0,4,7,10,14,21; n=4) with a MST of 16 days compared with a MST of 13.5 days in the control ($p<0.01$). The CD4-targeted treatments of MPA loaded nanoparticles (CD4+-

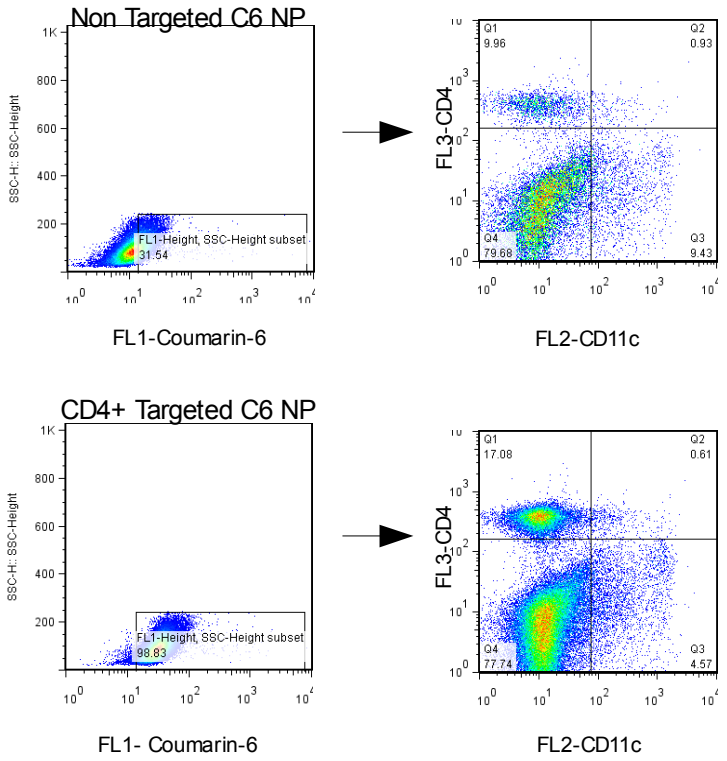

Figure 11. *Ex vivo* experiment where mice (n=3) were injected with treatments and 4 hours post injection splenocytes were harvested and stained for or CD11c-PE (FL2 channel), CD4-PerCP-Cy5 (FL3 channel) and Thy1.2-APC (FL4 channel). After negatively gating the FL1+ population from controls, FL1+ subgroups were examined for CD4 and CD11c. In the targeted nanoparticle group, a higher percentage of the FL1+ cells were CD4+CD11c- than in the non-targeted group.

NP-MPA, 0.5 ug anti- CD4+ Ab [clone RM 4-4] conjugated per mg NP-MPA, 5 mg CD4+-NP-MPA dosed per mouse on day 0,4,7,10,14,21; n=4) had an even greater delay with a MST of 25 days ($p<0.01$ compared with the control). The survival for the CD4-targeted nanoparticles was significantly greater than for the non-targeted particles (MST 25 days vs 16 days, $p<0.05$). The rejection in free MPA (5 mg per mouse dosed on day 0,4,7,10,14,21; n=4) and controls was statistically the same with a MST of 15 days ($p=0.06$ compared with the control). Mouse skin grafts showed rapid scabbing with eventual shedding of all grafts in all treatment groups (Figure 12).

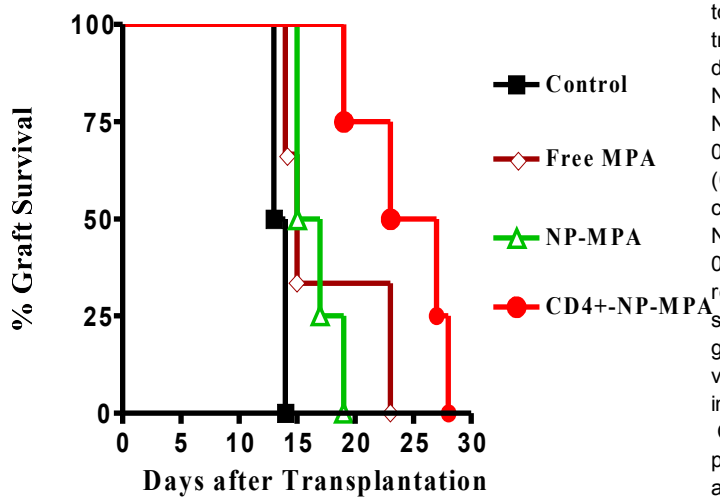

Figure 12. Full MHC mismatch BALB/C to C57BL/6 skin graft rejection in mice treated with free MPA (5 mg per mouse dosed on day 0,4,7,10,14,21; n=4), or NP-MPA (1ug MPA loaded per mg NP, 5 mg NP-MPA dosed per mouse on day 0,4,7,10,14,21; n=4) or CD4+-NP-MPA (0.5 ug anti- CD4+ Ab (clone RM 4-4) conjugated per mg NP-MPA, 5 mg CD4+-NP-MPA dosed per mouse on day 0,4,7,10,14,21; n=4). Control mice (n=4) received no additional treatment after skin grafting. Free MPA failed to improve graft survival over the control (MST 15 vs 13.5, p=0.06). Non-targeted particles improved graft survival (MST 16, p<0.01). CD4-targeting resulted in robust graft prolongation (MST 25, p<0.01 vs control) also greater than the non-targeted (p<0.05)

BALB/C to C57BL/6 MLR

To further characterize the phenotype that we observed with *in vivo* skin transplants, we determined lymphocyte proliferation via a recall antigen *ex vivo* MLR. We looked at differences between treatment groups (Free MPA 5mg/injection, CD4-targeted MPA nanoparticles 5mg/dose and non-targeted MPA nanoparticles at 5mg/dose)(n=4 per group) in post-transplant C57BL/6 CD4 enriched T cells stimulated with irradiated BALB/C splenocytes. Mice had their spleens harvested at 35 days post-transplantation. There was an equal and large decrease in proliferation as measured by radioactive thymidine uptake in both the CD4-targeted and non-targeted animals (mean counts per minute (CPM): CD4-targeted = 9627±36, non-targeted = 8812±696) compared with both the control (mean CPM = 24224±2169) and the empty nanoparticle (mean CPM = 20860±2083) ($p<0.05$ for both sets of comparisons) (Figure 13).

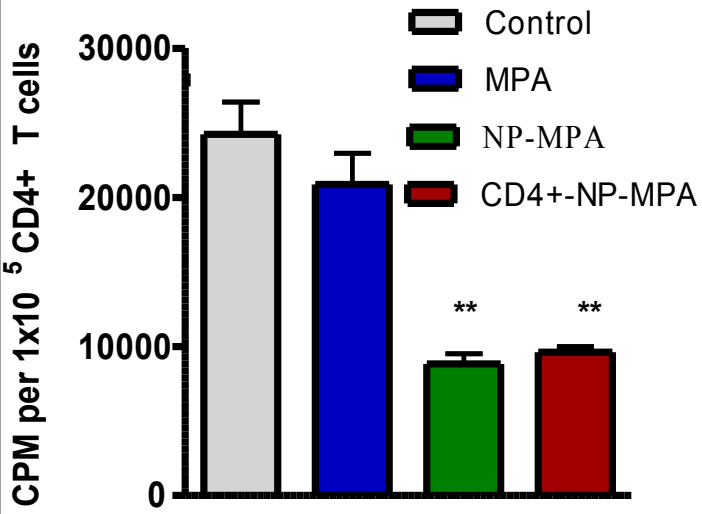

Figure 13. *Ex vivo* enriched CD4+ T cells isolated and pooled from mice (n=4) in each treatment group (Free MPA 5mg/injection, targeted and non-targeted 5mg/injection)on day 35 post-transplant were cultured in complete Bruff's media for 56 hours in 96-well plates at $1x10^5$ cells per well along with irradiated BALB/c splenocytes ($1x10^6$ cells per well). ^{3}H-thymidine was added for the last 18 hours and uptake was measured by a β plate scintillation counter. Mean CPM for each group: control-24224, empty NP-20860. Mean counts per minute (CPM) was statistically decreased ($p<0.003$ **) in both CD4-targeted and non-targeted treatments

We also looked at *In vitro* MLR with C57BL/6 CD4 enriched splenocytes cocultured with BALB/C irradiated splenocytes cultured for 72 hours in each treatment group (Free MPA at 10ug/ml, 1ug/ml, 0.1mg/ml; N-MPA and empty-NP at 0.1mg/ml and 0.01mg/ml and control)(n=3 per group) and found a significant decrease in proliferation in free MPA groups in a dose dependent manner (Figure 14) (MPA 10mg/ml mean CPM = 42.5±6.3, MPA 1mg/ml mean CPM =76.6±36.6, MPA 0.1mg/ml mean CPM =329±54.7) compared to the control group (mean CPM = 8708±840) ($p<0.05$ for each MPA dose vs the control). Nanoparticles loaded with MPA also showed a dose dependent decrease in proliferation (NP-MPA 0.1mg/ml mean CPM = 80.7±24.4, NP-MPA 0.01mg/ml mean CPM=1880±58.9) compared with the control ($p<0.05$ for each group). Empty nanoparticles showed a much greater proliferation response (NP 0.1mg/ml mean CPM= 5582±850, NP 0.01mg/ml mean CPM=7104±278) than the equivalent concentrations of MPA-loaded particle (NP vs NP-MPA at 0.1mg/ml, $p=0.003$; NP vs NP-MPA at

0.001mg/ml, p<0.0001) and a statistically similar proliferation with the controls (Figure 14).

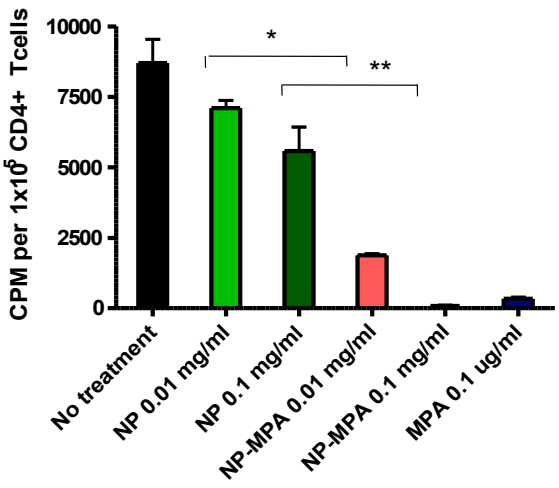

Figure 14. *In* vitro enriched splenic CD4+ T cells from naive B6 mice were co-cultured with irradiated BALB/c splenocytes in a one-way MLR without added treatment or treatment with free MPA, graded doses of empty NP (NP), or graded doses of NP encapsulated MPA (NP-MPA). NP were encapsulated with 6 µg of MPA per mg of NP. Compared to no treatment, empty NP had no significant effect on T cell proliferation at either dose of NP (NP 0.1 mg/ml, P=0.06; NP 0.01 mg/ml, P=0.14), whereas compared to no treatment, NP encapsulated MPA inhibited proliferation at both doses (NP-MPA 0.1 mg/ml, P=0.005; NP-MPA 0.01 mg/ml, P=0.001). For comparison of NP vs. NP-MPA, * denotes P=0.0001 and ** denotes P=0.003. Graph only shows one dose of free MPA as the other doses were similar and equally low

B6.H-2bm12 to C57BL/6 grafts

In order to further test and characterize the efficacy of CD4 targeting delivery of MPA, we transferred to a single MHCII mismatch CD4 dependent system for both *in vivo* and *in vitro* studies. We repeated skin grafts in B6.H-2bm12 to C57BL/6 mice (Figure 15). Both targeted CD4-NP-MPA (MST = 39days, p=0.0004) and non-targeted NP-MPA loaded (MST = 33days, p=0.002) nanoparticles show significantly delayed transplant rejection as compared with no treatment (MST = 19.5 days). Targeted (p=0.01) and non targeted MPA loaded (p=0.05) particles also show delayed rejection when compared to intermittent free MPA dosing (MST = 22 days). There was no statistical difference in graft survival between the targeted and non-targeted MPA groups. Daily free dosing (MST = 26 days) showed no significant difference in survival compared with the intermittent free MPA and both CD4-NP-MPA and NP-MPA. Daily free MPA delayed

rejection as compared with the control as well (p= 0.01). Targeted empty (MST = 18) and non-targeted empty nanoparticles (MST = 20) had similar graft rejection as compared with the no treatment group (Figure 15). In this less immunogenic model (as compared to BALB/C to C57BL/6), overall graft rejection was less rapid, but complete rejection was still found in all treatment groups.

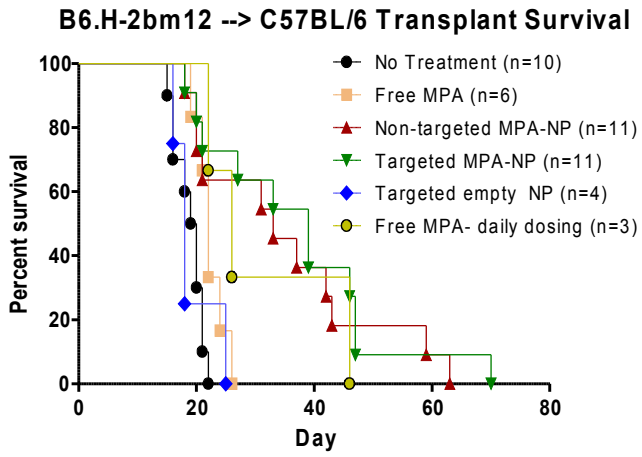

Figure 15. Skin graft survival in B6.H-2bm12 graft to C57BL/6 host mice, single MHCII mismatch model. Mice were treated with free MPA (5 mg per mouse dosed on day 0,4,7,10,14,21; n=6), or NP-MPA (1ug MPA loaded per mg NP, 5 mg NP-MPA dosed per mouse on day 0,4,7,10,14,21; n=11) or CD4+-NP-MPA (0.5 ug anti- CD4+ Ab (clone RM 4-4) conjugated per mg NP-MPA, 5 mg CD4+-NP-MPA dosed per mouse on day 0,4,7,10,14,21; n=11. Control mice received no additional treatment (n = 10). Mean survival times (MST) was significantly increased in both CD4-targeted (MST 39, p=0.0001) and non-targeted (MST 33, p=0.002)MPA-particles compared with NoRx mice (MST 19.5). Both targeted and non-targeted MPA-particles increased MST over intermittent free MPA (MST 22, p<0.05) but not over daily free MPA(MST 26). Targeted and non-targeted MPA-particles had similar MST. All controls had similar MST(18-20 days).

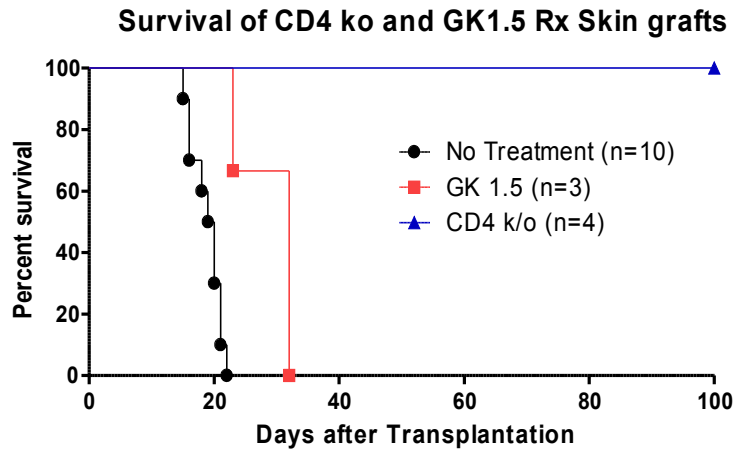

Figure 16. Skin graft survival data in B6.H-2bm12 graft to C57BL/6 host mice treated with PBS (control) B6.H-2bm12 graft to C57BL/6 host mice ttreated with GK1.5 depleting antibody, or B6.H-2bm12 graft to C57BL/6 -CD4 k/o host mice. CD4 k/o accepted grafts indefinitely. GK1.5 treated mice had statistically increased MST of 32 days over control mice MST of 19.5days (P<0.006).

CD4 k/o and GK1.5 Transplantation

Rejection in B6.H-2bm12 to C57BL/6 graft was shown to be a CD4, MHCII dependent process. We looked at skin transplants in a CD4 knockout mouse on a C57BL/6 background receiving a B6.H-2bm12 skin graft. CD4 knockout mice failed to reject their skin grafts, with eventual complete graft acceptance (Figure 16). Furthermore, in animals treated with GK1.5, an antibody to CD4 that inhibits function, we found delayed graft rejection (MST =32 days, $p<0.006$) as compared with control groups (MST 19.5 days). However all animals treated with GK1.5 eventually had complete graft rejection (Figure 16).

Rejection Histology

In order to observe the cellular response within the graft and the differences in response between treatment groups, skin graft biopsies were collected for histology on

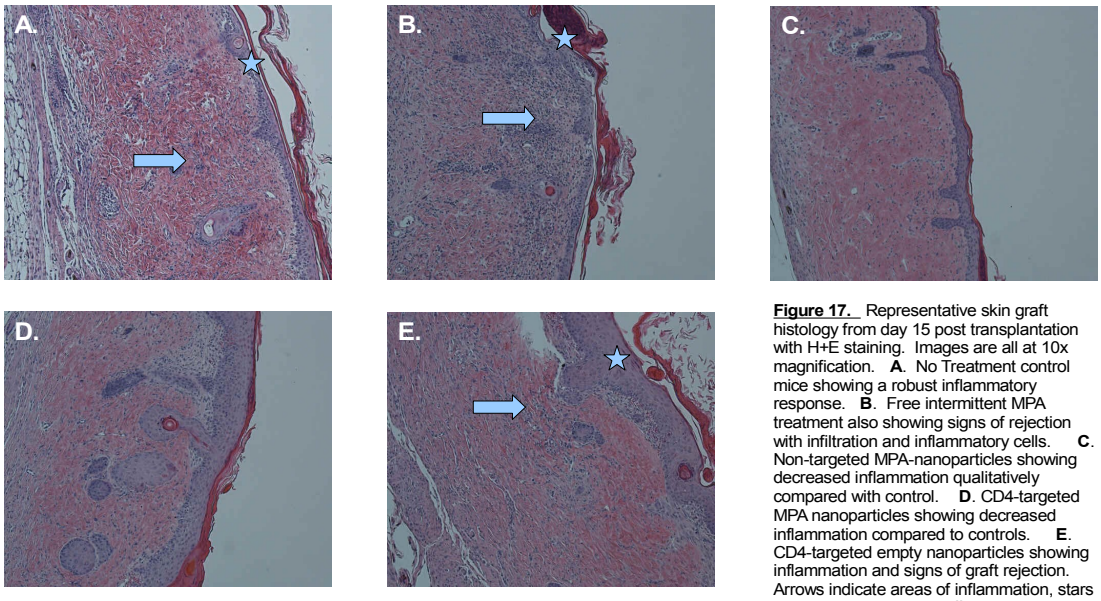

Figure 17. Representative skin graft histology from day 15 post transplantation with H+E staining. Images are all at 10x magnification. **A**. No Treatment control mice showing a robust inflammatory response. **B**. Free intermittent MPA treatment also showing signs of rejection with infiltration and inflammatory cells. **C**. Non-targeted MPA-nanoparticles showing decreased inflammation qualitatively compared with control. **D**. CD4-targeted MPA nanoparticles showing decreased inflammation compared to controls. **E**. CD4-targeted empty nanoparticles showing inflammation and signs of graft rejection. Arrows indicate areas of inflammation, stars indicate skin sloughing off.

day 15 from representative mice within each treatment group. Histology of the skin graft showed increased inflammation and cellular infiltration with increased signs of acute rejection. The targeted and non-targeted MPA-loaded nanoparticle treatment groups exhibited less lymphocytic infiltration and decreased overall signs of acute rejection (Figure 17). The observations were qualitative and no formal scoring system was used for comparison.

B6.H-2bm12 to C57BL/6 MLR

Lymphocyte proliferation and the effect of MPA and MPA-loaded particles was also examined via MLR and showed a decrease in proliferation after culture with MPA-

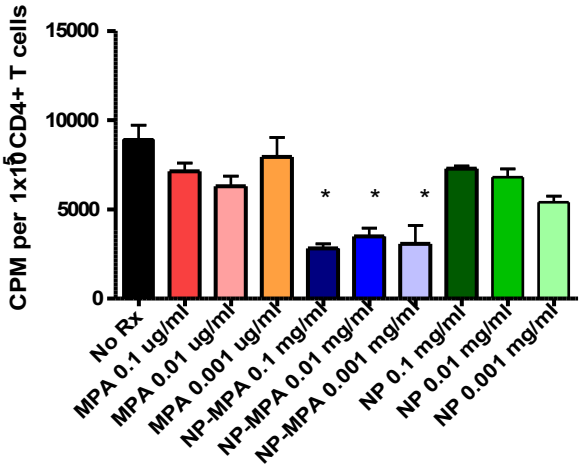

Figure 18. C57BL/6 splenocytes were CD4+ T cell enriched and cocultured in a one-way MLR with B6.H-2bm12 BMDCs that had been previously cultured for 24h with LPS, free MPA, empty NP or NP-MPA at increasing concentrations listed in the figure in 96-well plates at 1x10^5 cells per well along with the irradiated DC's (1x10^6 cells per well). 3H-thymidine was added for the last 24 hours and uptake was measured by a β plate scintillation counter. Mean CPM was decreased in all concentrations of nanoparticles loaded with MPA (NP-MPA) ($p<0.03$ * for each comparison) compared with control. NP-MPA at all concentrations was decreased compared with free MPA at all concentrations as well ($p<0.05$ * for each comparison).

loaded nanoparticles. Our i*n vitro* MLR used C57BL/6 splenocytes cocultured with B6.H-2bm12 bone marrow derived dendritic cells that had been been previously cultured with increasing free MPA concentrations, increasing empty particle and increasing MPA-loaded particle concentrations (Figure 18). NP-MPA at 0.1mg/ml (mean CPM

=2808±261, p<0.01), at 0.01mg/ml (mean CPM = 3484±457, p<0.02) and 0.01mg/ml (mean CPM =3071±1026, p<0.03) were all significantly decreased from the control (mean CPM = 8918±794). NP-MPA treated groups were also significantly decreased (p<0.05) from the free MPA groups (MPA 0.1mg/ml CPM = 7136±458, MPA 0.01mg/ml CPM = 6294±565, MPA 0.001mg/ml CPM = 7930±1098) (Figure 18). Due to a problem with the extraction, the *ex vivo* results were not interpretable for comparison.

Particle Uptake in Dendritic Cells

As we hyopthesized that dendritic cells were involved in the phenotype that we

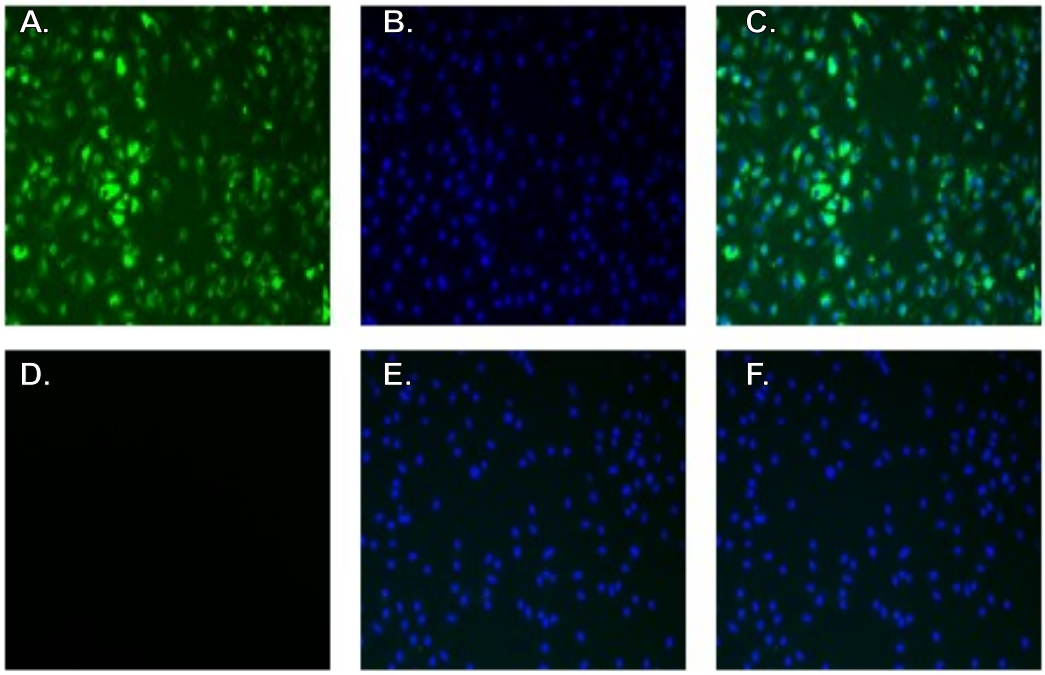

Figure 19. Incubation of cultured DC's collected from bone marrow with nanoparticles for 2 hours with images obtained at 20x magnification using a fluorescent microscope **A**. Shows coumarin-6 fluorescence in the green-fluorescent channel in loaded-nanoparticles incubated with DC's. **B**. DAPI counter-staining of the nucleus in the coumarin-6 loaded incubation. **C**. Overlay of green and blue channels coumarin-6 and DAPI staining shows nanoparticles in attached to cell surface, within the cytosol and possibly at different stages of phagocytosis. **D**. Green-channel fluorescence in DC's cultured with empty particle control. **E**. DAPI nuclear counter stain. **F.** Overlay of green and blue channel in empty particles.

observed with similar graft rejection between targeted and non-targeted nanoparticles, we wanted to see if DCs would take up nanoparticles while in culture. DCs were incubated on slides overnight for attachment. Uptake of nanoparticles was examined by comparing DCs cultured with coumarin-6 loaded particles with empty particles at 2 hours and 18 hours of incubation. At both 2 hours and 18 hours the coumarin-6 loaded particle-treated groups showed increased DC uptake of nanoparticles with increased fluorescent signal compared to the controls (Figure 19). Furthermore, the fluorescent signal appeared to be both diffusely increased in the cytoplasm as well as focally increased in nanoparticles attached to the cell membrane in what may appear to be varying stages of phagocytosis (Figure 19).

Cytokine Activation

To characterize the inflammatory response and the modulation that we expected to find with treatment using MPA-loaded nanoparticles, we also looked at cytokine profiles. The supernatant from MLR with culture of pre-treated DCs from B6.H-2bm12 mice with CD4 enriched C57BL/6 cells was analyzed for IL-2 production via ELISA. IL-2 production appeared to be decreased in a dose dependent fashion with increasing doses of MPA-loaded nanoparticles (N-MPA0.1mg/ml mean IL-2 concentration = 382±17 pg/ml, $p<0.005$; N-MPA 0.01mg/ml mean IL-2 concentration = 523±62 pg/ml, $p<0.02$; N-MPA0.001mg/ml mean IL-2 concentration = 636±6 pg/ml, $p<0.01$) compared to the control (Figure 20). Increasing doses of free MPA also showed decreased production in a dose dependent manner (MPA10ug/ml mean IL-2 concentration = 262±11 pg/ml, $p<0.004$; MPA1ug/ml mean IL-2 concentration = 661±10 pg/ml, $p<0.01$; MPA0.1ug/ml

mean IL-2 concentration = 695±24 pg/ml, p<0.02; MPA0.01ug/ml mean IL-2 concentration = 800±46 pg/ml, p<0.03) compared with the control (No Rx mean IL-2 concentration = 1251±57 pg/ml). (Figure 20). IFN-gamma levels (although we expected them to be low) were unchanged between treatment groups, nor did we find an increase in IL-10 production in the MPA-loaded particles (data not shown). Overall numbers were low in these experiments and were therefore difficult to interpret.

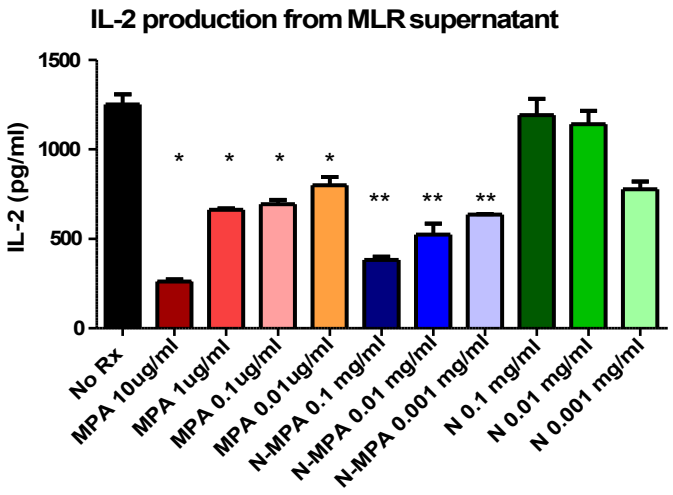

Figure 20. IL-2 production from MLR coculture of B6.H-2bm12 DC's at 1x10^6 cells/well with C57BL/6 CD4 enriched splenocytes at 1x10^5 cells per well after treatment of DC's with increasing doses of free MPA, nanoparticles encapsulating MPA and empty nanoparticles. Free MPA at all concentration showed a decrease in IL-2 production compared with control (p<0.03* for each comparison) N-MPA at all concentrations decreased IL-2 (p<0.02 ** for all comparisons).

In addition to the MLR cytokine production, we also looked at the profiles of activated dendritic cells in the presence or absence of NP treatments. DCs that were pre-treated with free MPA, empty particles, MPA-loaded particles and control groups were then stimulated with LPS and production of IL-6 and IL-12 was measured (Figure 21). IL-6 cytokine production was significantly decreased in N-MPA at all concentrations (N-MPA0.1mg/ml mean IL-6 concentration = 63738±4055 pg/ml, p<0.05; N-MPA0.01mg/ml mean IL-6 concentration = 53339±3664 pg/ml, p<0.05) compared with the NoRx +LPS control (mean IL-6 concentration = 132671±2295 pg/ml). Free MPA was also found to decrease IL-6 production (MPA9ug/ml mean IL-6 concentration =

40815±1661 pg/ml, p<0.01; MPA0.9ug/ml mean IL-6 concentration = 43483±1855 pg/ml, p<0.01) compared with the control. IL-12 was also reduced in one experiment by doses of N-MPA (N-MPA0.1mg/ml mean IL-12 concentration = 22306±141pg/ml, p<0.05; N-MPA0.01mg/ml mean IL12 concentration = 44919±871 pg/ml, p<0.05). Free MPA was also able to decrease IL-12 at 9mg/ml (mean IL-12 concentration= 36054±270 pg/ml, p<0.05) and 0.9mg/ml (mean IL-12 concentration = 54403±900 pg/ml, p<0.05) (Figure 21). We did not find any up-regulation of IL-10 in these tests either, and downregulation of TNF was also not seen (data not shown).

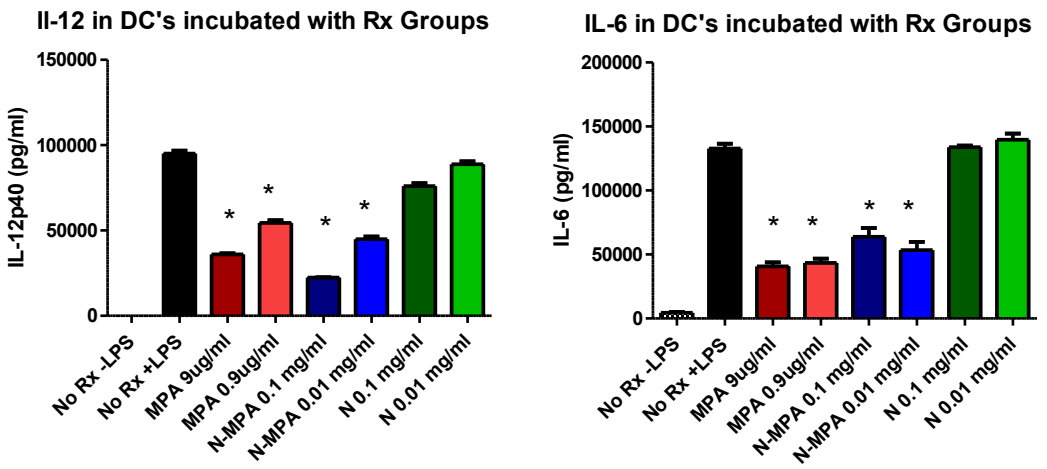

Figure 21. IL-12 and IL-6 production from DC's. DC's were collected from C57BL/6 bone marrow grown for 5 days, incubated with treatment groups for 24 hours, and subsequently stimulated with LPS for an additional 24 hours. Both free MPA and Nanoparticles encapsulating MPA showed a decreased production of both IL-12 and IL-6 compared with control (p<0.05 *).

DC Maturation Markers

As we thought that MPA-loaded nanoparticles may be affecting dendritic cells, we also looked for any changes in costimulatory and maturation surface markers on DCs

after treatment. Draining lymph nodes from transplanted mice were harvested on day 15 and cells were stained for DC maturation markers with CD40-FITC, CD80-FITC, MHCII-PE and CD86-APC. Cells were run on flow cytometry to determine differences in surface expression and gated on CD11c+ populations. CD40, CD80 and MHCII surface expression was unchanged in animals treated with free MPA, non-targeted-nanoparticles loaded with MPA and CD4-targeted nanoparticles (Figure 22). CD86 expression was

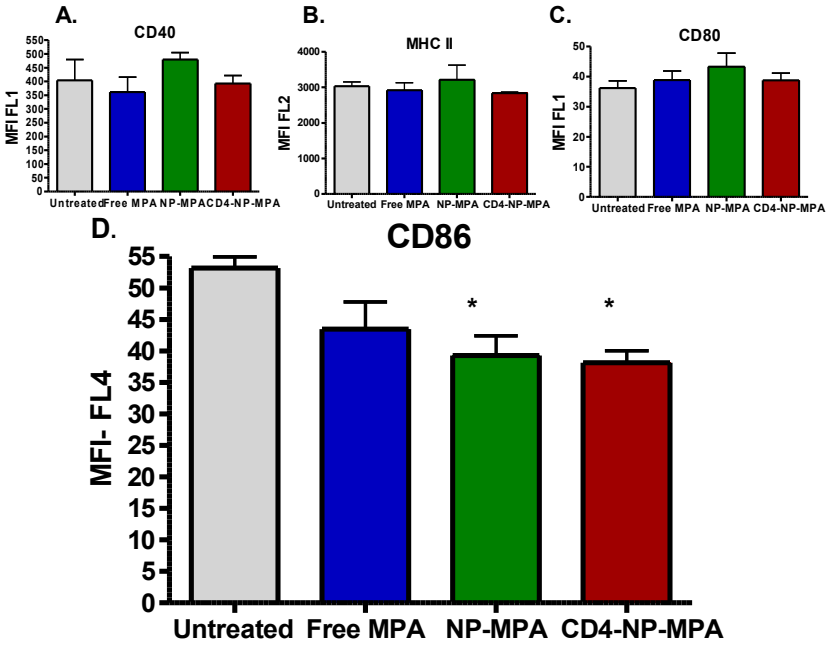

Figure 22. Draining lymph nodes were collected from mice on day 15 post-transplant. Lymphocytes were stained *ex vivo* with DC maturation markers CD40-FITC, MHCII-PE, CD80-FITC and CD86-APC. There were no difference in CD40 **(A),** MHCII **(B)** or CD80 **(C)** between groups. **D**. CD86 showed a significantly decreased CD86 surface expression via mean fluorescence intensity (MFI) with both targeted and non-targeted treatments (n=3, $p<0.05$) compared with controls (stars = $p<0.05$).

significantly decreased in both targeted (MFI = 38±2, $p<0.005$) and non-targeted (MFI = 39±3, $p<0.02$) MPA-loaded particle treatment groups (n=3/group) (Figure 22). *In vitro* DCs were also stained with CD80, CD86, CD40 and MHCII after 3 days of proliferation and subsequent 24 hours of treatment with free MPA, N-MPA and empty particles, and no differences were found in surface expression in the CD11c gated population (data not shown).

Treg proliferation

Regulatory T cells have been shown to be involved in decreased immune responses to transplantation (64), so we also looked for increases in Tregs after treatment with MPA-loaded nanoparticles. At day 21 post-transplant we collected splenocytes from our treatment mice (Free 5mg MPA, targeted and non-targeted N-MPA at 5mg particle) and stained the cells *ex vivo* with CD4-APC (FL4 channel), CD25-FITC (FL1 channel) and *Foxp3*-PE (FL2 channel). We gated on side and forward scatter for

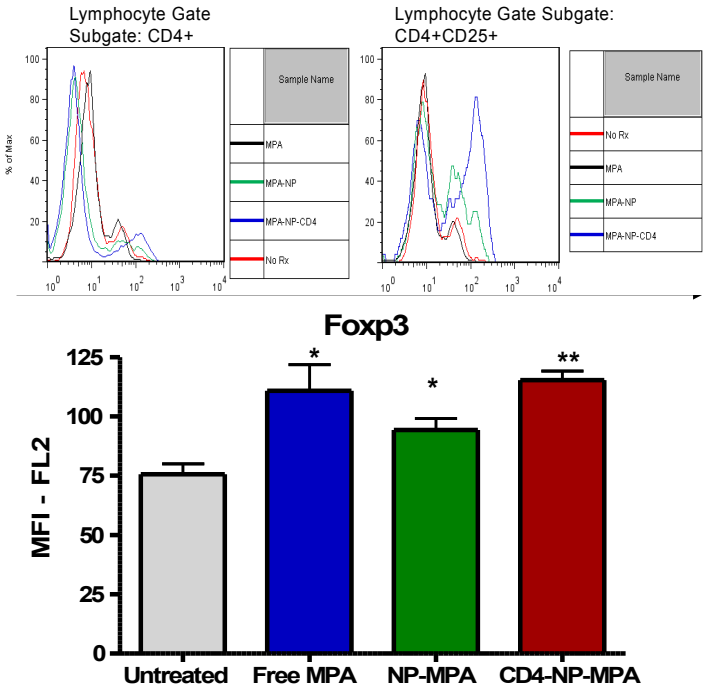

Figure 23*Ex vivo* splenocytes were collected at day 21 post-transplantation and were stained with CD25-FITC, CD4-APC and*foxp3*-PE Lymphocytes were gated with FSC and SSC, and *foxp3* (FL2) mean intensity (MFI) was compared between treatment groups (n=3/group). Free MPA and NP-MPA showed significant increases in fluorescence ($p<0.05$ *) and CD4-NP-MPA showed highly significant increase in fluorescence ($p<0.003$**)

lymphocytes and looked at the CD4+CD25+ population of cells and compared mean fluorescence intensity of *Foxp3* (FL2). We found an increased MFI in free MPA (MFI =

111±11, p<0.05) in non-targeted particles (MFI = 94±5, p<0.05) and in CD4-targeted particles (MFI = 115±4, p<0.003) (Figure 23).

Apoptosis Flow Cytometry

To determine if there were increases in dendritic cell death through apoptosis, we collected DCs after incubation with our treatment groups for flow cytometry. We stained DCs with 7-AAD (FL3 channel), Annexin (FL1 channel) and CD11c-APC (FL-4). We gated on a DC population using forward and side scatter and subgated on the FL4 channel to capture CD11c+ cells, and determined relative percentage of cells undergoing apoptosis in each group (Figure 24). Apoptotic cells were determined as 7-

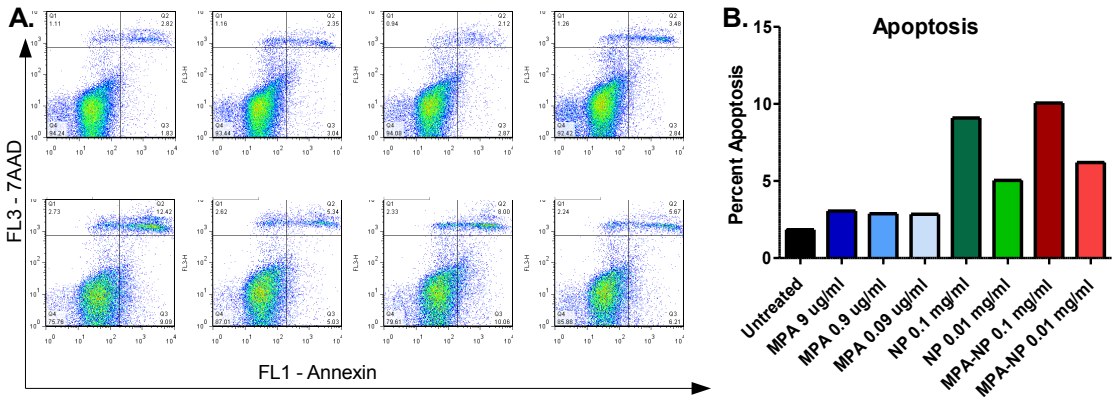

Figure 24. DC's incubated with treatment groups were collected and stained with annexin (FL1), 7AAD (FL3) and CD11c-APC(FL4). Populations were gated on DC population via SSC vs FSC and gated further on CD11c+ populations. Apoptotic cells were considered 7AAD negative, Annexin positive. A. Diagram showing FL1 vs FL3 channels. B. graph of percentage of cells from the third quadrant of every treatment group showing increased apoptosis in N-MPA groups. (n= 1 for each group)

AAD negative, Annexin positive. There was more apoptosis in both MPA loaded (10% 7AAD-Annexin+) and empty nanoparticles (9% 7AAD-Annexin+) at high concentrations

(0.1mg/ml) without sufficient data to run statistics (n=1 per group) (Figure 24).

B7-DC and B7-H1 Flow Cytometry

B7-DC and B7-H1 have been shown to be important negative co-stimulatory molecules that may be involved with dendritic cell decrease in T cell antigen response. We were interested to determine if these molecules were involved with the phenotype that we observed in our system. In DCs that were pretreated with MPA-loaded particles,

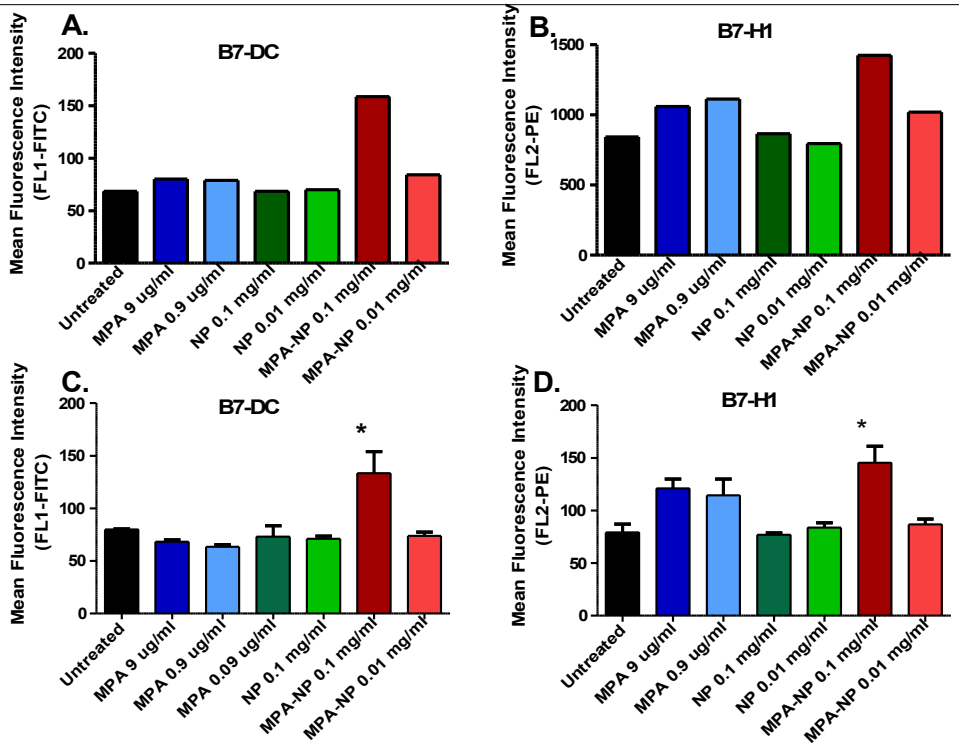

Figure 25. Mean fluorescence intensity of DC's stained with B7DC-FITC and B7H1-PE. A,B. Results from first experiment (n=1 for each group) with increased expression in the 0.2 mg/ml MPA-NP group. C,D. Experiment was repeated with two different mice showing a consistent increased expression of both B7-DC and B7-H1 on DC's treated with MPA-loaded nanoparticles (at 0.1 mg/ml) and gated on CD11c+ population. In the second experiment, B7-DC and B7-H1 fluorescence were both statistically increased compared with the control group ($p<0.05$ *). Data was not combined between the first and second experiments due to difference in gating and staining.

empty particles, free MPA and no treatment, we found consistently elevated mean

fluorescence looking at B7-H1 and B7-DC surface expression in groups treated with 0.1mg/ml MPA-loaded particles. Our initial experiment (n=1) showed this trend in both B7-DC (NP-MPA0.1mg/ml fluorescence = 158, control fluorescence = 68) and B7-H1 (MPA0.1mg/ml fluorescence = 1424, control fluorescence = 842) (Figure 25). Repetition of the experiment with an additional two animals confirmed the results with significant increase in surface expression of both B7-DC (MFI = 134±21, $p<0.05$) and B7-H1 (MFI = 146±16, $p<0.05$) compared with the control (Figure 25).

Discussion

Summary

MPA-loaded particles showed a slow and continuous release profile *in vitro* and biodistribution demonstrated a possible increased nanoparticle retention in the spleen. We showed effective targeting of nanoparticles to CD4 T cells using both *ex vivo* and *in vitro* analysis by flow cytometry. In the fully allogeneic MHCII mismatch BALB/C to C57BL/6 mice we found improved graft survival in the non-targeted MPA group and even greater graft survival in the CD4-targeted group, as well as decreased proliferation with both targeted and non-targeted nanoparticles using *in vitro* and *ex vivo* mixed lymphocyte reactions. In the less immunogenic B6.H-2bm12 to C57BL/6 transplants, a single MHC-II mismatch model, we found equal and increased graft survival in both the CD4 targeted and non-targeted animals. In both the highly immunogenic and less immunogenic models, graft survival times were increased over free drug using the nanoparticle encapsulation and the total dose of drug given to attain this increased survival was roughly one thousand fold lower than the free drug concentration. We also showed that the B6.H-2bm12 to C57BL/6 model was CD4 dependent by administration of the depleting antibody GK1.5 and by transplanting skin from a B6.H-2bm12 mouse onto CD4 knockout mouse bred onto a C57BL/6 background. It was postulated that the similar rejection times in targeted and non-targeted groups was due to dendritic cell (DC) involvement and we found active uptake of nanoparticles in DCs, a decrease in inflammatory cytokine production and a decrease in treated DCs ability to stimulate T cells via mixed lymphocyte reactions. Furthermore we found a possible mechanism of nanoparticle-MPA action on the DC interaction with T cells through the upregulation of the inhibiting co-stimulatory molecules B7-DC and B7-H1 on DCs treated with MPA-

nanoparticles. These ligands bind the PD-1 receptor on activated T cells and have been implicated in a decreased immune response. We also found possible upregulation of CD4+CD25+ *Foxp3* expressing T cells which may serve to increase graft acceptance. These results indicate the feasibility of nanoparticles loaded with immunosuppressive drugs to be used clinically and illustrate the involvement of dendritic cells in the process of nanoparticle induced graft acceptance.

B6.H-2bm12 to C57BL/6 Survival

The results of our graft survival show that CD4-targeted and non-targeted MPA loaded particles significantly delay graft rejection by two to three weeks over the control groups. The results also showed that targeted particles do not significantly delay graft rejection over non-targeted particles despite a mean survival time increase of an additional six days in the B6.H-2bm12 to C57BL/6 transplants. It is possible that the study was under-powered to show subtle differences between these two groups, but it is also possible that there is no actual difference between targeted and non-targeted nanoparticle treatments with both groups working very well to delay transplant rejection.

The intermittent free drug was significantly less effective at decreasing graft rejection as compared with the MPA-loaded particles. Mycophenolate mofetil in its oral form exhibits peak blood concentrations of MPA at 1-2 hours after conversion in the liver to its active form. It also exhibits a mean half-life between 9-17 hours, with great inter-subject variability. Because of this, it is generally dosed twice a day in humans (65). Therefore, our intermittent free drug treatments were large doses quickly cleared by the mouse. Due to this clearance, there were multiple days where MPA blood levels were

likely very low until the next treatment with MPA. We did not examine the pharmacokinetics of MPA in our mice, but we thought that the intermittent dosing was not an optimal comparison to our nanoparticles despite the thousand fold lower dose in the nanoparticles. To address this we included in our study a daily intermittent dosing in one of our later transplant groups. Due to the low number of animals in this treatment group we did not achieve a statistically significant difference in mean survival time compared to either the intermittent free MPA or with either of the MPA encapsulated nanoparticles, even though the MST was four days longer than the intermittent free drug and 13 days shorter than the MST for targeted nanoparticle treated groups. Nevertheless, it is interesting that daily free drug in doses that are more than a thousand fold greater STILL do not show improved graft survival than the nanoparticle delivered MPA. This is consistent with literature showing that nanoparticle encapsulated drugs can be conveyed with better efficacy due to the delivery characteristics of the nanoparticles (59). Furthermore, our results agree with literature showing that single dose injections of PLGA nanoparticles encapsulated with drug can have equal *in vivo* efficacy as daily injected doses of the drug (42). Some studies have even found improved efficacy of single dose PLGA nanoparticle injections as compared with multiple free drug injections (66). This delay in graft rejection is also clearly not secondary to effects of the empty nanoparticles alone as empty particles that are targeted and non-targeted show the same mean survival time as the non-treated group. In addition, the histology confirms the robust inflammatory response seen in the control groups and the decrease in cellular infiltration and inflammation in the nanoparticle-MPA treatment groups with delayed rejection.

BALB/C to C57BL/6

It is also interesting that in our small data set using BALB/C to C57BL/6 skin transplants, we found significantly greater graft survival in the targeted MPA-nanoparticle group when compared to the non-targeted MPA-nanoparticle group. This difference might represent the small numbers of animals in each group and be due to insufficient power, but it may also represent a different phenotype as compared with the less immunogenic B6.H-2bm12 to C57BL/6 transplants (as the difference was statistically significant with a $p<0.05$). In skin-grafted mice, the migration of MHC class II donor-derived dendritic cells (DCs) to the recipient's lymph nodes triggers a potent direct CD4 T cell alloresponse. Skin grafts can be rejected directly or indirectly by both CD4 and CD8 cells activated against both MHC and minor antigens in a major mismatch model such as a BALB/C to C57BL/6 skin transplant. Interestingly however, one group that looked at B6.H-2bm12 to C57BL/6 skin grafts found only the direct allopresentation response in their model (62). This is of interest within our study due to the phenotypic difference in rejection between the highly immunogenic BALB/C to C57BL/6 and the less immunogenic B6.H-2bm12 to C57BL/6 graft. If the highly immunogenic model relies upon both direct and indirect allorecognition, and the indirect response is absent from the less immunogenic B6.H-2bm12 to C57BL/6 model, than perhaps this explains the observations in the targeted and non-targeted groups in both models. This has interesting possible implications as it may suggest that targeted nanoparticles in the BALB/C to C57BL/6 affect the indirect pathway to greater degree (increasing MST in targeted treatments) and with this presentation pathway absent in the B6.H-2bm12 transplants, perhaps this contributes to the similarity between targeted and non-targeted

groups. This interaction has not been examined in the literature, but it may be worth further study. To test this you could utilize models that isolate either the direct or indirect pathways, and compare targeted and non-targeted nanoparticle treatments within each model. These models may be created using transgenic mice (such as CD11c-DTR transgenic mice that can be conditionally depleted of CD11c+ DCs with administration of diphtheria) that impair DC function/antigen presentation (back-crossed or bred on BALB/C, C57BL/6 and B6.H-2bm12 mice) as both donor and recipient mice. The indirect pathway may be isolated using our current models with depletion of donor DCs within the skin graft before prior to transplantation. These may be interesting avenues of study because if the indirect pathway is affected more by targeted nanoparticles, this could have implications for the elusive treatment of chronic rejection, as the indirect pathway has been implicated in chronic rejection pathogenesis(17).

MLR interpretation

Interestingly, despite the difference in targeted and non-targeted treatments *in vivo* of graft survival times in the BALB/C to C57BL/6 mice, the *ex vivo* MLR on these animals showed a reduction in proliferation that was equal between the targeted and non-targeted groups. Although the flow cytometry data likely indicated successful targeting with CD4-targeted particles, it is unclear how well targeting actually induces a down-regulation of alloimmune cells, as the decrease in proliferation was equal in the *ex vivo* targeted and non-targeted groups. This finding may also simply represent a difference between a transplant response and a MLR in the behavioral response to MPA-loaded particles and be an artifact of the assay itself. Unfortunately, the *ex vivo*

data from our B6.H-2bm12 transplants were not interpretable after technical problems during the radioisotope extraction, so we are not able to compare *in vivo* to *ex vivo* results in the B6.H-2bm12 transplants. The *in vitro* data from both transplant models are consistent showing a decreased proliferation with MPA-loaded particles. Unfortunately, we were not able to test the specific interaction of nanoparticles with CD4 T cells in our MLR as CD4 T cells remain in suspension and cannot be separated for subsequent culture with dendritic cells and splenocytes. In the future we may attempt to flow-sort the sample to isolate T cells from suspension (as long as T cells are not non-specifically activated by flow sorting). In our BALB/C model the co-incubation of irradiated splenocytes, CD4 T cells and treatment groups likely showed particle interactions with both CD4 T cells and the APCs in the splenocytes. Initially we thought that irradiation of the splenocytes would decrease phagocytosis and lead to a response that was reliant upon the CD4 T cell uptake of nanoparticles, but there does not appear to be any literature that clearly shows that APC irradiation decreases phagocytosis, and therefore the response we found is likely due to nanoparticle interaction with both cell types. In our B6.H-2bm12 *in vitro* MLR's we found a response that was dependent strictly on pretreatment of DCs. We cultured the nanoparticles with DCs before coculture with CD4 T cells which points to the involvement of DCs in our *in vivo* skin graft rejection phenotype. Our MLR's therefore were able to show nanoparticle affects on the proliferative response through DCs but were not able to show an affect through CD4 T cells alone. Overall however, the results of these MLR's are consistent with our graft survival data, and consistent with the observed *in vitro* changes in cytokine expression with an overall decreased inflammatory and proliferative response.

CD4 dependence and targeting

It was important to determine the CD4 dependent nature of the B6.H-2bm12 to C57BL/6 model, to make sure that the effect that we saw from treatment was secondary to CD4 T cell activity. Using GK1.5 depleting antibody against CD4, we saw a modest increase in graft survival although a change that was less than expected (still statistically significantly greater than the control). It is possible that the doses of antibody used were insufficient to decrease a proliferative response, and that a larger dose of GK1.5 would have increased mean graft survival longer. Furthermore with only three animals in this group, we may have had too few animals to properly see the larger difference. Using a CD4 knockout mouse as the recipient allowed for acceptance of the graft showing the CD4 dependent rejection in our model. Since CD4 T cells regulate both the cytotoxic and humoral allospecific response of CD8 and B-cells, the CD4 knockouts likely have reduced overall immunity leading to graft acceptance. The results of both our GK1.5 and CD4 knockout mice are in agreement with a study looking at pancreas xenografts from rat to mouse that was shown to be CD4 dependent similarly to our model but in a different organ system and model. GK1.5 treatments delayed graft rejection, while CD4 knockout mice showed acceptance of the graft in a model that suggested dependence on CD4 cells for rejection (67).

The discovery that non-targeted nanoparticles loaded with MPA displayed equal skin graft prolongation as the targeted particles in our B6.H-2bm12 to C57BL/6 mice was initially a surprise. It is unclear if this lack of difference was due to poor targeting of the nanoparticles. Targeting was suggested with the flow cytometry with increased FL1 channel signal in the targeted coumarin-6 particles as compared with the non-targeted

particles. The *ex* vivo data were not shown to be significant however due to the small number of samples (with one outlier) and this needs to be repeated to truly prove targeting. Despite this lack of significance, in the targeted nanoparticle group the FL1 coumarin-6 positive cell population had an increased percentage of cells in the CD4+CD11c- subpopulation. In the non-targeted nanoparticle treatment, the FL1 coumarin-6 positive cell population had an increased population in the CD11c+CD4- subpopulation. The increased number of coumarin-6 fluorescent cells in the CD4+CD11c- population with targeted nanoparticles indicated that specific binding/uptake in CD4 cells was likely greater than the non-specific/binding/uptake in DCs expressing CD11c. In addition our collaborators have consistently found targeting with these same particles in their previous studies using anti-CD3 (48)(32) and in their unpublished data showing success with anti-CD4 targeting as well. One question that remains unclear though (as it does with most flow cytometry comparisons) is how any observed difference in fluorescence would actually translate into a meaningful clinical or functional difference. The data from our collaborators comparing retro-orbital injections of PEGylated rhodamine loaded PLGA particles compared with CD4 targeted particles showed increased retention in the spleen after injection in the targeted particles, but this data is difficult to interpret as it was only done in one mouse/group, and the CD4 targeted particles appeared to be retained in all organs to a greater degree than the non-targeted particles. These data would need to be repeated to determine any significance. Furthermore a difference in biodistribution alone would not prove targeting, but the large CD4 population in the spleen would explain any increased retention compared with non-targeting. The lack of difference in biodistribution that we found at three hours post intraperitoneal injection may be due to retention in the peritoneum (as opposed to direct

introduction into the vasculature with retro-orbital injections) and a longer time may be needed post injection to be able to find systemic differences in nanoparticle retention. Overall, our data suggests successful targeting, but needs to be repeated to determine the consistency of these findings.

Dendritic Cell Involvement

We postulated that our targeted and non-targeted nanoparticles may be exhibiting two different mechanisms abrogating graft rejection and increasing mean survival times of skin grafts in our B6.H-2bm12 model. The CD4-targeted particles likely increase transplant acceptance through their activity directly on CD4 T cells via cytosolic delivery of MPA while the non-targeted particles induce acceptance through some other pathway that was initially unclear to us (with CD4-targeted particles likely acting through this pathway to some degree as well). The flow cytometry data from our *ex vivo* animals showed increased FL1 signaling and hence increased binding/uptake of the non-targeted particles in the CD11c+CD4- subgroup that makes up the dendritic cell population. This indicated to us that dendritic cells possibly were actively taking up the non-targeted nanoparticles and suggested to us that the non-targeted particles may be yielding their effects through activity on the dendritic cells. As DCs are important scavengers and sentinels of the periphery with enormous influence on the fate of T cell populations (68), we speculated that uptake of nanoparticles into dendritic cells may be involved in our observed phenotype.

It was suggested in one study that dendritic cells exert a powerful influence in skin graft transplant models due to the higher than normal numbers of dendritic cells that

are resident within skin compared with other tissues (62). Dendritic cells in general have a great influence on transplant rejection, which has consistently been shown throughout the transplant literature. The direct and indirect antigen presentation pathways have been shown to be vital in presentation of foreign antigen and development of an allogenic immune response (15) . Dendritic cells exert their influence on the rejection process in multiple different ways, and are the important front-line cells in the detection and allospecific response to foreign antigens (68). It has been shown that the peak size of the allospecific T cell population is an important factor in the speed and robustness of the rejection (69). It has also been shown that an important factor in the peak size of this T cell population is the number of antigen presenting cells that are activated (70), with larger numbers of activated DCs leading to a larger T cell response. Overall, dendritic cells increase the transplant response through their direct and indirect activation of T cells, presentation of costimulatory molecules and production of excitatory cytokines (15).

Dendritic cells have also been shown to be involved in the down-regulation of the transplant rejection immune response. This down-regulation could be through their absence, decreasing the number of activated antigen presenting cells and subsequently decreasing the alloreactive T cell population, but DCs have also been shown to directly down-regulate the immune response as well. This action is through their production of ligands for negative costimulation, decreased activation of T cells with changes in cytokine profiles and induction of tolerant anergic responses via immature dendritic cells and up-regulation of CD4+CD25+ regulatory T cells (71).

MPA uptake via nanoparticle delivery may lead to inhibition of dendritic cells which in turn would lead to decreased activation of graft specific T cells. Our microscopy

of dendritic cells cultured with nanoparticles showed robust phagocytosis of the particles by dendritic cells. Furthermore, we found a decreased mixed lymphocyte reaction response with B6.H-2bm12 dendritic cells treated with MPA-nanoparticles cocultured with C57BL/6 CD4 cells. This *in vitro* response indicated that dendritic cells were likely less able to activate T cell proliferation after the DCs had been exposed to MPA-nanoparticles. Qualitatively it was also noticed that the DCs treated with the nanoparticles appeared to be less healthy in appearance and formed fewer clusters in culture which may have been an indication of decreased function, and decreased ability to activate a T cell response. In addition, we found decreases in inflammatory cytokine expression in DCs cultured with MPA-particles. With regard to the *in vivo* rejection response, these cumulative data suggested that DCs may have been responsible.

MPA and Dendritic Cells

Recently it was shown that dendritic cells are inhibited by exposure to MPA. In one study the investigators found that immature human DCs exposed to MPA were induced to a mature phenotype yet showed a decreased allogeneic T cell activation through both the direct and indirect pathways (72). Another study suggested that MPA was able to decrease the functional maturation of dendritic cells, finding both decrease maturation markers as well as functional decreases in inflammatory cytokine production (73). As dendritic cell trafficking to lymph nodes for antigen presentation is an important aspect of the development of the acquired immune response, one group has been looking at the affects of MPA on dendritic cell trafficking in blood samples taken from healthy human volunteers. They found that MPA decreased the expression of

chemokine receptor 7 (CCR7) and increased the expression of CCR1 (upon maturation DCs suppress CCR1 and switch to increased expression of CCR7) which correlated with a decrease in DC homing to lymphatic tissues (as CCR7+ cells are drawn by chemotactic gradient towards lymphatic tissues) thus inhibiting a crucial step in the initiation of an alloimmune response. They also found a generally immature phenotype of DCs treated with MPA, impaired activation via TLR3 ligation and decreased T cell activation via MLR (74).

Our results examining cytokine production indicated that our DCs after treatment with MPA and MPA-loaded particles could be involved in the down-regulation of the immune response. We found decreases in IL-6 and IL-12 in our free MPA, and nano-MPA treatments after stimulation with LPS consistent with the literature showing a decrease in inflammatory cytokines produced by DCs after treatment with MPA. Similarly to our study, Lagaraine et al. and Mehling et al. also found decreases in IL-12 production by their DCs treated with MPA. As IL-6 production has been shown to require p38MAPkinase (75), and p38MAPkinase has been shown to be inhibited by MPA (76), our decrease in IL-6 that we found is understandable. Consistent with this as well were our results showing decreased IL-2 production in our MLR supernatants in the groups with DCs pretreated with MPA and MPA-nanoparticles, which was also consistent with the decrease in proliferation via MLR that we found in these treated groups.

Similarly to Lagaraine et al., after culturing DCs with MPA loaded nanoparticles we did not find a shift to an immature phenotype as we found no differences in surface MHC, CD80 or CD86 expression via flow cytometry either *ex vivo* or *in vitro*. Our collaborators in Dr Fahmy's laboratory, however, while using a similar nanoparticle (modified from our PLGA particles) examined coculture with DCs from an early time

point starting at day 1 of DC culture and in the MPA-loaded particle group they were able to find a shift in the maturity phenotype to an immature form with decreased surface expression of CD40, CD80 and CD86 (unpublished data). As their particles were slightly different in structure from ours and they started incubation at Day1 instead of Day3 or Day5 like we did in our experiment, the change that they found may be due to either of these factors and we are planning on recapitulating the data using an earlier time point for culture. Our *ex vivo* data are difficult to compare with the *in vitro* data obtained by other investigators. Our results however are similar to Lagaraine et al. who found decreased activation with MPA treated DCs (similarly to us) but in the context of a mature phenotype (72). The difference in maturity phenotype may be related to the time point of DC exposure to MPA. Lagaraine et al. similarly to our study did not begin exposure of DCs until 5+ days after beginning the culture, and they did not find any change in maturity phenotype. However, both Mehling et al. and Cicinnati et al. found an overall decrease in DC maturity but both of their studies cultured their DCs with MMF or MPA from day one of DC culture (73) (74). Consistent with the literature, the difference in our results are likely explained by the timing of the treatment, but we would need to repeat the *in vitro* study to determine if this is in fact true.

Overall, the literature indicates that MPA pushes the immune system towards decreased immunity through interactions not just with T and B cells but through interactions with antigen presenting cells causing decreased antigen presentation, decreased direct and indirect alloimmunity, decreased APC function and increased overall graft acceptance. It remains unclear however the mechanism by which MPA exerts these effects on DCs. As MPA inhibits IMPDH, this has been examined as a possible pathway within the DCs. One group found that MPA decreased p38MAPKinase

by 25% (DC maturation is highly dependent on p38MAPK). However, exogenous guanosine failed to reverse the effects of MPA in DCs leading the researchers to speculate that MPA exerted its effects on DCs independent of IMPDH (76). The mechanism however remains elusive.

Regulatory T cells

Regulatory T cells (Tregs) are a group of T cells that have an immunomodulatory role in the development of antigen-specific immune responses. There was initially a great deal of controversy as to to existence and role of Tregs after early research in the 1970's led to their suggestion (77). In the early 1990's however, a group found that the observed transplantation tolerance in their study could only be due to Treg involvement (78). This paper furthered the idea of "infectious" tolerance that had been suggested by Gershon et al. in 1971, and Qin et al. found that the transfer of CD4 T cells from tolerant mice were able to induce tolerance in naive lymphocytes and that these newly tolerant lymphocytes were able to subsequently induce tolerance in another naive group; hence their "infectious" nature (78). This work has since caused an explosion in research looking at Tregs with much focus on Tregs in transplantation. The most recognized and best studied group of Tregs are "natural" Tregs produced in the thymus that are CD4+CD25+ and express *Foxp3* which is a transcription factor that is essential to Treg growth and differentiation (79). The development of CD4+CD25+ Tregs has been shown to be dependent upon the cytokines IL-10 (80) and TGF-beta (81). Tregs appear to modulate transplant rejection with their ability to induce antigen specific tolerance, with upregulation of CD4+CD25+ T cells that are specific to alloantigen decreasing the graft

specific immune response (64). Multiple pathways have been explored to determine the nature of this interaction and DCs appear to both affect and be affected by Tregs (71). DCs have been shown to be capable of expanding Treg CD4+CD25+ positive populations (82) and Tregs have been shown capable of decreasing maturation and functionality of DCs (83).

There has also been work looking into the interaction of MPA and DCs with Tregs. One study using porcine dendritic cells found low expression of B7 costimulatory molecules, low levels of IL-12 production and generally weak proliferative mixed lymphocyte reactions when DCs were cocultured with peripheral blood mononuclear cells (PBMC's). They also found that after multiple exposures to MPA treated DCs, PBMC's inhibited the alloproliferative response likely indicating an induction of Treg cells capable of modulating the alloimmune response (84). Another study has shown that DCs treated with MPA increased the antigen specific Treg population possibly increasing the level of tolerance to antigen specific responses (85).

As the literature also suggests that MPA and DCs can contribute to the formation of Tregs, we examined this interaction in our model as well. We looked for expression of cytokines that lead to Treg production such as IL-10 (86), but were unable to detect any differences in production between groups. We plan to measure TGF-beta, which is a classic stimulator of Treg formation (86) as well. Despite no differences in cytokine production in our treatment groups *in vitro* that would push differentiation to Treg formation, our flow cytometry data with staining for CD4, CD25 and *Foxp3* showed a likely proliferation of Tregs in our MPA treatment groups. We need to repeat this in more mice for confirmation, but the experiment indicates a statistically significant increase in fluorescence for Tregs in the CD4-targeted animals, free MPA and non-targeted MPA-

nanoparticles when comparing mean fluorescence intensities. It remains unclear why we may have an increase in Treg development in the absence of increased cytokines as the literature looking at DCs and MPA pushing a Treg phenotype finds a parallel increase in IL-10 and TGF-beta (85), but perhaps this difference is due to our testing conditions. Our increase in Tregs was found *ex vivo* roughly three weeks post-transplant, while our *in vitro* testing for cytokine up-regulation was via MLR, DCs treated with MPA and nanoparticles for 24 hours and a short DC/CD4 co-culture without secondary stimulation after co-culture. Lagaraine et al. in 2008 found an up-regulation of Treg inducing cytokines using a 5-day coculture MLR with secondary re-stimulation with anti-CD3/CD28 as well as an extended length 4-week coculture, and in both shorter and longer experiments the cultures were treated continuously with MPA (85). Our lack of IL-10 cytokine expression therefore is not surprising considering the much shorter duration of interaction between DCs and CD4's and the decrease in nano-MPA treatment times in our *in vitro* experiments compared with the literature.

Role of PD-1 and PD-1 Ligands

The role of co-stimulation in T cell activation has been well established in the development of the alloimmune response (87). The CD28:B7 costimulation is the most well understood of these co-stimulatory interactions and has been shown to be involved in the initiation of T cell responses (88)(89). To counter-balance these “excitatory” signals, the body has the ability to negatively co-stimulate as well. CTLA-4 which is induced after T cell activation also binds to B7 on APCs and has been shown to be an important inhibitor of T cell function (90). The role of CTLA-4 is exhibited dramatically in animals that are CTLA-4 knockouts as these animals die rapidly from massive

lymphoproliferation (91).

Our discovery of up-regulation of B7-DC and B7-H1 in our mice treated with MPA loaded nanoparticles was a very intriguing finding. The programmed death-1 (PD-1) receptor is a recently uncovered molecule that exhibits negative regulation of T cells. It is a member of the CD28 family and was cloned from T cells undergoing apoptosis (92). Unlike other members of the CD28 family, PD-1 has been found on CD4, CD8, B-cells as well as myeloid cells, but is especially prevalent on activated T cells (93). Two ligands for PD-1 have been discovered, PD-L1 (B7-H1) and PD-L2 (B7-DC) both in the B7 family but distinct from B7-1 and B7-2 that are involved in T cell costimulation. PD-L1(B7-H1) has been found on activated APCs such as dendritic cells, monocytes and B cells (94). Induction of expression on peripheral APCs has been found secondary to exposure to inflammatory cytokines, while PD-1 expression is found on predominately on activated T cells (95). Thus, the interaction between this receptor-ligand appears to be critical in regulation of effector and memory T cells with local modulation of specific T cell responses as sites of inflammation.

The role of PD-1 in tolerance has also been clearly shown with its expression on the placenta during pregnancy and its location in a position that might help facilitate protection against the mothers' immune system (96). In addition, both PD-L1(B7-H1) knockout mice and mice treated with antibody blocking PD-L1 rejected semi-allogeneic pregnancies but accepted syngeneic pregnancies (97), further illustrating the importance of the PD-1 pathway in immunogenic tolerance. PD-1 has been shown to be an important regulator of immunity especially within autoimmunity, with multiple autoimmune murine genetic lines showing PD-1 involvement in the development of disease entities such as lupus, diabetes, autoimmune cardiomyopathy and

encephalomyelitis (98)(99)(100)(101).

The PD-1 pathways has been strongly implicated in modulation of the T cell response. In one study examining PD-1 involvement in heart graft rejection, PD-1 appeared to down-regulate alloreactive T cells. The same study also showed that blockade of PD-L1 (B7-H1) increased the speed of allograft rejection, further solidifying the role in T cell modulation (102). In another study by this same group using an adoptive transfer of T cells antigenically specific to the mutated MHC class II molecule I-Abm12 (our B6.H-2bm12 mice), they found that PD-L1(B7-H1) blockade enhanced T cell proliferation, inhibited allospecific T cell apoptosis and skewed the response to a Th1 phenotype (103). PD-1 has also been implicated with Tregs, as one study found a decrease in Treg numbers in PD-L1(B7-H1) knockout mice (104). Another study found that blockade of the PD-1:PD-L1(B7-H1) pathway abrogated Treg immunoregulation suggesting that the pathway is necessary for Treg function and upregulation (105). Recently, a group in Boston demonstrated that PD-L1(B7-H1) has a pivotal role in the regulation of induced Treg development as well as the maintenance of the Treg response. They found that PD-L1 (B7-H1) knockouts are only able to minimally induce Tregs, and that PD-L1(B7-H1) coated beads are able to induce Tregs. In addition they found that PD-L1(B7-H1) increases the expression of *Foxp3* and sustains the immunosuppressive function of the cells, clearly implicating PD-1 and its ligand B7-H1 as centrally important in Treg maintenance and function (106). Numerous other studies have also implicated PD-1 as an important part in the upregulation of Tregs. This connection is intriguing within our study as we have found both an increase in Tregs, as well as an upregulation of the PD-1 ligands B7-DC and B7-H1 in our nano-MPA treated groups. The literature strongly supports a connection between these separate findings

and our graft rejection phenotype may be due to both an upregulation of the PD-1 pathway through enhanced expression of the PD-1 ligands B7-DC and B7-H1 with a possible subsequent Treg proliferation. Despite this information however, it is not completely clear how PD-1 and the PD-1 ligands regulate T cell function, and is likely not through a single pathway, but through a combination of apoptosis, induction of anergy and immunoregulation.

Interestingly, evidence is building that the PD-1 receptor is not the only receptor involved in the effects of the PD-L1, and binding effects may be more wide spread. In vivo blockade of PD-1 results in different effects then blockade of PD-L1. Sandner et al. showed that blockade of PD-L1 significantly decreased the amount of apoptosis, but blockade of PD-1 failed to decrease the apoptosis response (103). Another study suggested that PD-L1 in tumor cells was able to induce apoptosis of T cells specific to the tumor in a non-PD-1 mediated manner (107). Uncovering these other receptors may be valuable in further understanding how the PD-1 ligands B7-H1 and B7-DC are fully involved with modulating the immune response.

PD-1 and MPA

Recently, a group published a report uncovering a connection between the programmed death ligand system and treatment with MPA (108). They observed that the PD-1:PD-L interaction in T cells inhibits cytokine production, proliferation and results in cell cycle arrest. They also observed that the affect of MPA on dendritic cells was similar and hypothesized that this novel receptor-ligand interaction might be involved in the behavior seen in DCs treated with MPA. In agreement with previous work, they found

that MPA decreased the allostimulatory behavior of DCs in a dose dependent manner. They also found a down-regulation of costimulatory/maturation molecules including MHCII, CD80 and CD86 also consistent with previous reports, and they found a decrease in Th1 profile cytokines (108). This decrease in maturation markers with the early treatment of DCs with MPA is consistent with the results found by other investigators that treated DCs with MPA early in their growth (73) (74). Interestingly, Geng et al. also made the novel finding that MPA treated DCs upregulated PD-L2 (B7-DC) also in a dose dependent manner, without any difference seen in PD-L1 (B7-H1) (108). While this finding does not prove causation, it certainly suggests possible involvement of the PD-1 inhibitory pathway in the observed effects of MPA on DCs and their role in modulation of T cell activation. In related studies, it has been reported that MPA affects MAPkinase pathways independent of IMPDH pathways in DCs (76). A different group found that PD-L2:PD-1 interaction upregulated MAPkinase in T cell's (109). Therefore, the interactions between the MAPkinase pathways, PD-1 and MPA, may be an interesting avenue of exploration to determine the connection between MPA and PD-1.

Our findings showing up-regulation of B7-DC and B7-H1 in our MPA-nanoparticle treated dendritic cells are therefore consistent with the results of Geng et al. and the delay in transplant rejection that we found in our non-targeted group may be due in part to the up-regulation of these negative co-stimulatory molecules secondary to the effects of the MPA nanoparticles. We also found a small increase (not significant) in apoptosis and cell death with flow cytometry staining for annexin and 7-AAD, although these results would need to be repeated to determine if they are consistent, significant and not an artifact of the particles themselves (as empty particles also showed an increase in

this population). However, if this increase apoptosis is real, it may be secondary to the MPA treatment as it has been suggested in previous studies that found increased apoptosis in DCs treated with MPA (110)(72). It remains unclear the mechanism of action that may cause induction of apoptosis as MPA may affect both the IMPDH pathway and other unknown pathways (76). However, increased apoptosis may also be through the B7-DC and B7-H1 pathways, as these have also been shown to be involved in the induction of apoptosis in other immune cell types (107). Overall, the expression of PD-L1 (B7-H1) on a dendritic cells appears to be a functionally relevant and important pathway in the development of tolerance to transplantation (71), and its interaction with immunosuppressive medications and use to induce transplant acceptance will be a valuable direction for further research .

Role of Nanoparticles in Transplant

Much of the research investigating nanoparticle modulation of transplant rejection by other investigators has limited translatability and comparability to our study, as the models that other studies have employed are dramatically different from skin transplantation. The corneal transplant models used by multiple researchers looking at encapsulated tacrolimus, sirolimus and cyclosporine are generally avascular, and are likely very different in physiology from other transplantations. It is difficult therefore to extrapolate from these studies to other forms of transplantation. The susceptibility of T cell mediated transplant rejection has been shown to be very different depending on the type of graft tissue and the location of the graft placement (111). Indeed, corneal transplants and skin transplants represent the extreme ends of allogenicity and

immunogenicity. It has been shown countless times in the C57BL/6 – BALB/C model that mice will rapidly reject their skin transplants over the course of 1-2 weeks, and that this rejection is only moderately modifiable with general resistance to transplant modification treatments. Skin allografts are therefore considered the most immunogenic of all transplanted tissues (16).

In contrast, the corneal transplant in these same mice is very well tolerated without any treatment, and roughly 50% of grafts are spontaneously accepted, with the remaining grafts slowly rejected over 8-10 weeks. Furthermore, this slow rejection is easily modifiable using immunosuppressive agents (16). This sensitivity to immunosuppressive agents may be due to the fact that the cornea itself is poorly immunogenic and the eye is a site of immune privilege (112). It has been shown in a study comparing skin and corneal transplants that the skin transplants reject through both direct and indirect pathways by both CD4+ and CD8+ cells activated against both MHC and minor antigens. Corneal transplants however, only reject via CD4+ T cells that are indirectly activated against minor histocompatability antigens (16). If Schenk et al. are correct that our B6.H-2bm12 mice reject through only a direct pathway (62), then the skin transplants used in our study would respond in a dramatically different fashion from a corneal transplant.

The comparison of our research to the work of Alemdar et al. using dopaminergic CNS transplantations (55)(56), exhibits similar problems for relation to our study as the corneal transplant research. The central nervous system is also an immune privileged site similarly to the eye (113) and transplant rejection and tolerance is likely mediated by factors that are different from the highly immunogenic allogenic skin graft. The CNS produces a number of anti-inflammatory molecules which decrease the overall

inflammatory response (113). MHC expression on resident immune cells has been shown to be relatively low, decreasing T cell activation and proliferation. The blood brain barrier maintains this protection so despite its vascularization it is relatively protected from the rest of the body (113). Overall it is difficult to determine the applicability of the findings of Alemdar et al. compared with our system because of both the type of transplanted tissue as well as their use of liposomal particles (not PLGA like in our study).

The comparisons to these other models illustrates a potential limitation of our work in translation to clinical allografts. Similarly to the difference between rejection of corneal and CNS transplant and skin transplants, the biology of skin graft rejection may also be different from solid organ vascularized grafts used clinically such as the heart, kidney, lung and liver. Skin appears to be the most immunogenic transplanted graft, with lung and small bowel also causing a robust immune response. In decreasing immunogenicity are pancreatic islets, vascularized pancreas, heart, kidney and liver transplants (114) (111). Liver allografts are sufficiently non-immunogenic to be accepted without treatment in many animal models (115). Our model with skin allografts which initiate the most robust immune response may therefore not represent the same response that might be seen in other tissues. In skin transplants which are not vascularized, the histologic findings of acute and chronic rejection involving immune invasion and deposition within the vascular walls will not be found. Non-vascularized grafts are also more susceptible to ischemic damage (116) leading to non-specific inflammation and necrosis which may allow the graft to become more affected by the targeted immune response. This increased sensitivity however has been largely dismissed as evidence has mounted that vascularized skin grafts also reject with similar

intensity as non-vascularized grafts (111). The mechanism behind the increased immunogenicity of skin allografts remains unclear but other possibilities have been proposed including the presence of tissue specific antigens in the skin. The skin also serves as an immunologic barrier and thus has large numbers of dendritic cells with subsequent ability to robustly activate alloreactive T cells. Recent work showed that highly immunogenic skin grafts tend to require a lower number of effector T cells for rejection when compared to other solid organs like heart transplants (69)(62).

The work that is most directly comparable to our study was done by McAlister et al. who used liposomal tacrolimus to treat both skin allografts and heart allografts. They compared oral free vs oral liposomal drug and found improved skin graft survival with the liposomal formulation and unchanged survival of the heart allograft (58). Ultimately, this study is also difficult to compare with our own because this study delivered drug orally and used liposomal particles instead of PLGA particles, but overall it is in agreement with our results finding that nanoparticle encapsulated drug can be superior to free drug in highly immunogenic skin transplantation.

Due to these differences in graft behavior, future studies could move to one of two models that we have had experience with in our lab. The pancreatic islet transplant is less immunogenic and technically can be done with relative ease. Our lab has recently started using this model in aging studies (unpublished data), and this less immunogenic model might be able to tease out differences between CD4-targeting and non-targeted nanoparticles that we were unable to see in our skin graft model. An even greater improvement to our model would include using vascularized heart allografts. These transplants require far more technical expertise, surgical skill and time than the skin transplants we are currently employing, but this model would represent a human

solid organ transplantation much better than either a skin or pancreatic islet transplantation. We have had some experience in our lab with these transplants as well (117), and perhaps this would be a worthy future direction to consider.

Another area that would further strengthen the argument for use of nanoparticles in transplantation modulation would be to study targeting of nanoparticles encapsulated with other drugs such as tacrolimus, sirolimus and cyclosporine. Mychophenolic acid is an effective drug that has largely replaced azathioprine, but it is generally only used in combination with other medications as maintenance therapy, is rarely used alone and rarely for induction of immunosuppression (21). MPA is generally taken orally as the pro-drug mycophenolate mofetil and is actually fairly well tolerated compared with many other immunosuppressive medications. Therefore, MPA may not ultimately be the best drug for this technology to be used with. Multiple groups have had success already with rapamycin (49) cyclosporine (52)(50) and tacrolimus (53) encapsulation in PLGA particles for use in corneal transplants. Our collaborators have indicated that encapsulation of these drugs would be possible in their laboratory as well and this may present another interesting future approach to our work.

As clinically used immunosuppressives are given orally, it would also be interesting for us to develop an oral form of targeted nanoparticles. Oral formulations may be preferential in terms of compliance, ease of use and ability to treat away from a hospital or clinic. Using oral nanoparticles would be superior to using intravenous treatments, and this would be a valuable direction to take our research in the future. Oral preparations of liposomal drugs have been used to many years (doxirubicin, vincristine, amphotericin), and McAlister et al. even looked at oral liposomal tacrolimus (58), but there has been little work looking at oral PLGA encapsulation of

immunosuppressive drugs. One group has used PLGA particles encapsulated with cyclosporine as an oral treatment (40). Cyclosporine, due to its high molecular weight, rigid structure and the P-glycoprotein intestinal cellular efflux mechanisms (P-gp efflux) present in the GI system causes overall low bio-availability with oral intake. Nanoparticle encapsulation bypasses the P-gp efflux, decreases gastrointestinal tract degradation and gut wall metabolism and improves pharmacokinetic profiles and bio-availability. Nanoparticles can also allow protection from first pass liver metabolism as happens with traditional oral drugs (40). This group found that nephrotoxicity was decreased in the nanoparticle group and that the nanoparticle formulation exhibited consistent and controlled slow release with better bio-availability and intestinal uptake (40). These data indicate that PLGA particle oral formulations may have a viable future in transplantation and that it may be interesting to use oral formulations of MPA loaded particles in our transplant model.

Conclusion

Despite the little previous research that has been done looking at nanomedicine in transplant, it appears that this field has great potential and that nanoparticles will likely be an important avenue for the improvement of drug delivery. The medical management and treatment of patients post-transplantation is currently limited by the efficacy and dose-toxicity of the currently available immunosuppressive medications. With the considerable expense of developing new medications, the possibility of encapsulation of currently used drugs in nanoparticles with improved pharmacokinetics, local drug delivery and improved side effects make this avenue an attractive option. A bright future

of medicine lies in targeted therapies that deliver drug locally to specific tissues or cell types and spare the rest of the body from the deleterious effects, and targeted nanoparticles will likely be part of this future. Our research sets the stage for these therapies as our work is the first antibody or ligand targeted nanoparticle research in a transplant model. Our work suggested that targeted and non-targeted MPA-loaded particles are both capable of increasing graft survival at thousand-fold lower doses *in vivo,* and that MPA-loaded particles decrease lymphocyte proliferation with MLR, finding possible differences in efficacy of targeting dependent upon the immunogenicity of the model. We also found that targeting particles likely show specificity to CD4 T cells. Furthermore, we examined the mechanistic effects of our particles within our model with results suggesting dendritic cell involvement with uptake of MPA-loaded particles and subsequent decrease in the alloimmune response with up-regulation of Tregs, upregulation of the inhibitory costimulatory molecules B7-DC and B7-H1, and decreased inflammatory cytokines with MPA-loaded nanoparticles. Overall our research is a starting point to develop further clinically relevant nanoparticle-drug combinations, and further uncover and investigate the basic pathways and interactions underlying nanoparticle modulation of the transplant immune response.

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