CONTROLLED RELEASE FORMULATIONS FOR IMMUNO-REGULATION: *TOWARDS A SYNTHETIC TOLEROGENIC DENDRITIC CELL*

by

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TOWARDS A SYNTHETIC TOLEROGENIC DENDRITIC CELL

Siddharth Jhunjhunwala, PhD

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Transplant rejection and autoimmunity are characterized by adverse inflammatory reactions and the absence of immuno-regulatory mechanisms. One of the most common treatments for these conditions is the use of immunosuppressive agents that non-specifically suppress immune cell function. However, these agents are associated with many drawbacks that include renal and hepatic toxicities as well as increased risk of infections and malignancies. A promising alternative to immunosuppressants is the utilization of the immune system's natural regulatory mechanisms. Cellular therapies, comprising of tolerogenic dendritic cells (tDC) and regulatory T cells (Treg), which exploit these regulatory mechanisms are currently under development. Unfortunately, therapies that use tDC and Treg are associated with many challenges such as; difficulty in isolating these cells, problems in maintaining their regulatory phenotype under *ex vivo* culture conditions, and the prohibitive infrastructure requirements in culturing these cells under 'GMP' conditions. A potential solution to these problems is the development of prospective therapeutics that would alter regulatory cell numbers and function *in vivo*. To this end, we developed three new degradable and biocompatible formulations to modulate regulatory immune responses. The first formulation involves the encapsulation of an immusuppressant, rapamycin, into appropriately-sized microparticles (rapaMP) that can specifically be targeted to phagocytic cells *in vivo*. RapaMP-treated DC show lowered expression of co-stimulatory markers and decreased ability to stimulate T cell proliferation, both

of which are suggestive of their capacity to suppress immune responses *in vivo*. The second formulation involves the encapsulation of a chemokine capable of Treg recruitment (CCL22) into sustained release vehicles (CCL22MP). Our data demonstrates that CCL22MP are able to recruit Treg *in vivo*, and show promise in delaying cell and composite tissue graft rejection. The third formulation involves the encapsulation of factors (IL-2, TGF-β and rapa; FactorMP) that we have identified as agents capable of Treg induction. We show that FactorMP are capable of inducing functional Treg populations from both mouse and human cells, and could possibly create a local immunosuppressive environment *in vivo* that favors Treg proliferation. Controlled release formulations such as these, could potentially be developed as "off-the-shelf" therapeutics for the treatment of transplant rejection and autoimmunity.

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1.0 INTRODUCTION

Transplantation refers to the procedure of transferring cells and tissues from one location in the body to another (auto-transplantation) or from one body (donor) to another (host). This donor tissue may be of human (allo-transplantation) or non-human (xeno-transplantation) origin. The process of transplantation has been practiced for centuries, with some of the earliest reports dating as far back as 600 B.C. when the Indian surgeon Sushruta performed skin auto-transplantation for facial reconstruction [1]. Over the past few decades, allo-transplantation of solid internal organs has become common due to the advent of modern immunosuppressive drugs and improved surgical techniques [2]. Examples of some of the most commonly transplanted solid organs include kidneys, liver, lung and heart. The scientific registry of solid organ transplant recipients estimates that, at present, over 100,000 patients are living with a functioning transplant in the USA, and each year around 20,000-30,000 patients are added to this list [3]. Although the field of solid organ transplantation has grown considerably, it is still plagued with the problem of "*rejection*". Rejection is the response of the host's immune system against a donor allo-transplant, which is naturally recognized as foreign/non-self tissue [2, 4]. To understand the concepts of transplant rejection, one must first learn how the immune system works. The next section briefly describes the immune system and its components.

1.1 THE IMMUNE SYSTEM

A tremendous number of parasites and other infectious agents constantly threaten to invade and cause harm to organisms such as humans. Preventing the entry, proliferation and colonization of these pathogenic organisms is by no means a simple task. For this reason, the mammalian body has developed a complex immune system for defending itself from such infections. The primary goal of this system is to kill or sequester any foreign agent that enters the body, and also prevent proliferation and spread of abnormal self tissue (tumors) [5]. The integumentary system (skin, nails etc.) and the epithelial lining of the respiratory and digestive systems passively assist the immune system in preventing the entry of pathogens, while a number of cellular and acellular components of the immune system actively take part in neutralizing the agents that have made it past the initial entry barrier. The major components of the immune system can broadly be divided into the innate immune system and the adaptive immune system.

1.1.1 Innate Immune System:

Upon successful entry through the passive barriers, infectious agents first encounter the components of the innate system. For this reason, the innate immune system is often referred to as the first active line of defense. The cells of this system distinguish pathogen-associated cellular material from self tissue using a variety of discriminating features that can broadly be defined as pathogen or danger associated molecular patterns (*PAMPs and DAMPs*) [5-6]. Following identification of a pathogen, the innate immune system establishes a cascade of responses which are briefly described below (detailed description of this process can be found in reference [5]).

Tissue resident macrophages are the first cells of the immune system that encounter the invading pathogen. Macrophages as their name suggests, are phagocytic cells that are capable of ingesting entire microbes and killing (oxidizing) them in their endosomes / phagosomes. In addition to ingesting and neutralizing microbes, macrophages recognize PAMPs through cellular receptors on their surface. This recognition event leads to cellular activation and secretion of danger signals (cytokines and chemokines), which establish an inflammatory environment at the site of microbe entry. As Janeway and colleagues discuss [5], establishing an inflammatory environment is necessary for combating infection as; (i) it is the primary means by which immune cells are recruited to the site of microbe entry, and (ii) it allows for the creation of a physical barrier (through coagulation of blood vessels) that prevents the spread of the invading microbial agent.

Circulating neutrophils respond first to these inflammatory signals. They assist macrophages in ingesting invading microbes, and help to augment the inflammatory response. Many other cells of the innate immune system, such as eosinophils, basophils and mast cells can also be recruited to the site of infection, depending on the type of pathogen and tissue. The inflammatory mediators (mainly IFN-β, TNF-α and IL-12) also help to recruit and activate Natural Killer (NK) cells which are important in clearing away infections caused by intracellular pathogens.

Most importantly, professional antigen presenting cells known as **dendritic cells (DC)** [7] are also recruited to the site of microbial entry/infection. DC ingest “antigens” (whole microbes or cellular material from infectious organisms) and migrate to the lymph nodes where they can activate naïve T cells in an antigen-specific manner. Due to their unique capability of activating naïve T cells and initiating adaptive immune responses, DC are often referred to as the

bridge that connects the innate and the adaptive immune system. The process of DC-based T cell activation is very briefly described below (Figure 1) [5]. Antigens taken up by DC are digested inside phagosomes, and antigen fragments are presented on the cell surface attached to major histocompatibility (MHC) receptors. The antigen-MHC complexes bind to the T cell receptor (**signal 1**) leading to T cell activation. For strong, effective and stable activation, the DC must also express co-stimulatory molecules (CD80, CD83, CD86 among others), which bind to the CD28 receptor on the T cell surface (**signal 2**). Further, DC secrete soluble mediators (cytokines) that promote activation and differentiation of T cells (**signal 3**). The activation of T cells is the first step towards establishing a strong adaptive immune response against the invading pathogen.

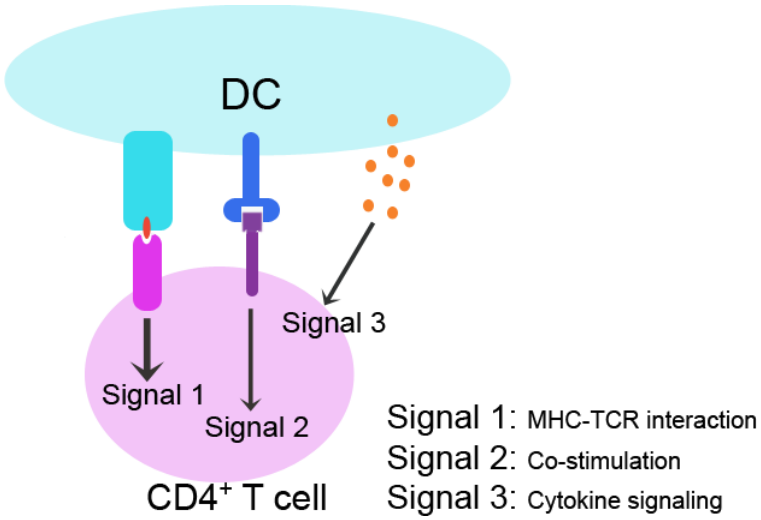

Figure 1: Cartoon describing DC-T cell interaction [5].

1.1.2 Adaptive Immune System:

The adaptive immune system, as its name suggests, has the remarkable ability to "adapt" itself to generate antigen/pathogen specific immune responses. The resulting highly specialized immune responses allow the body to fight against pathogens that have developed methods to bypass the innate immune system. T and B lymphocytes (as the mediators of adaptive immunity) are able to generate literally billions of different cell surface receptors (T cell receptors (TCR) and B cell receptors) through a genetic recombination process providing a way to recognize an equally diverse number of antigens expressed on the surface of various pathogens [8]. Upon interaction with DC that display a "*specific antigen"* coupled to their MHC receptors, *"antigen-specific"* T and B cells clonally expand and initiate *antigen-specific* adaptive immune responses in order to neutralize the corresponding *antigen-bearing* pathogen. Furthermore, a key characteristic of the adaptive immune response is its ability to generate immunological memory. Following the completion of the immune response against a pathogen, a select number of activated antigen-specific T and B cells survive to become *memory cells*. This "memory" allocation allows the body to respond to the same antigen faster than if the antigen were to be encountered anew. The details of this particular type of immune response will not be discussed in this dissertation (and can be found elsewhere [5]). However, the cells that form the adaptive immune system are described briefly below.

B and T lymphocytes are constantly generated from hematopoietic stem cells in the bone marrow, and there is believed to be a rapid turnover of these cells under immune homeostasis. B cells arise and mature in the bone marrow, where cells expressing self-reactive receptors are deleted. Following maturation, B cells for the most part reside in the lymphoid systems until the time they are activated. Upon activation these cells secrete antibodies against the specific

antigen, which is known as the humoral immune response. T cells in turn are also named after their site of maturation – the **T**hymus. Similar to B cells, T cells that express self-reactive receptors are deleted in the thymus, but differ from B cells in their function following activation. The two most well described and widely studied classes of T cells are: (i) helper T cells (express the CD4 receptor) – assist in establishing antigen specific immune responses through various means, such as activation of the CD8 expressing T cells, B cells, NK cells and macrophages; (ii) cytotoxic T cells (express the CD8 receptor) – directly take part in killing an infected cell or pathogen. This dissertation will mainly focus on the $CD4^+$ T cells, especially on a subset of these cells that are known to regulate the immune system.

$CD4^+$ helper T cells can differentiate into different subsets depending upon the activating conditions presented by the DC (signal 3 from Figure 1) during the process of activation. Broadly $CD4^+$ T cells may be characterized into 4 different classes, Th1, Th2, Th17 and regulatory T cells (Treg), although other minor sub-classes of cells have been reported in literature [9]. The phenotypical and functional characteristics of Treg will be discussed in later in this chapter (Section 1.10).

The next section will briefly describe the role of innate and adaptive immune cells in rejecting a transplant.

1.2 REJECTION IMMUNE RESPONSES

The immunological nature of transplant (graft) rejection was first discovered by Medawar and colleagues [4, 10], although at that time it was unclear as to what components of the immune system played a role in these responses. Over the past 50 years, immunologists have described

the immunological processes involved in rejection in great detail (reviewed in [11]). The following section will briefly summarize some of the main components of these complex immune responses against an allograft.

Transplantation surgery is associated with significant trauma as the diseased/dysfunctional tissue needs to be detached from the connecting blood vessels and nerves and new transplanted tissue reattached. This trauma acts as a "danger" signal [12] that initiates innate immune responses. Tissue transplantation is followed by a series of acute inflammatory episodes that are mainly directed by tissue resident macrophages, DC, invading neutrophils, and NK cells of the innate immune system [12]. Inflammatory mediators secreted by these cells can cause significant damage to the graft. Additionally, NK cells can take part in the killing of graft cells due to MHC mismatch. Further, the inflammatory environment can activate graft-associated antigen presenting cells (APC) to initiate a corresponding adaptive immune response.

Adaptive immune responses against a transplant can rise from two different types of antigen presenting cells. During the acute rejection phase (<6 months post transplant), it is believed that donor derived DC [13] (cells from the graft) are the most important stimulators of naïve T cells. The process of donor derived DC (which express distinct MHC molecules from host cells) acting as primary APC is called *direct antigen presentation* [13]. Interestingly, it has been shown that a large percentage of naïve T cells (~10%) can be primed by these MHC mismatched DC [12]. Such large numbers of activated T cells directly take part in graft destruction and also enhance other immune responses against the graft. For example, $CD4^+$ T cells initiate delayed type hypersensitivity responses by macrophages. Additionally, $CD4^+$ T cells can also activate B cells to secrete graft-specific antibodies. Adaptive immunity can also be initiated by host DC that present graft associated antigen to naïve T cells, a process called

indirect antigen presentation [13]. The immune responses initiated by host DC have not reported to be different from those generated by donor DC. However, it is believed that host DC play a much greater role in chronic rejection responses (>1 year post transplant) presumably following the destruction of all donor-derived DC [12].

Treating rejection has been a challenge, given the complicated nature of immune responses against the graft and the involvement of the many of the same cell types that actively protect us from infections. The next section will focus on some of the current clinical treatments for suppressing rejection responses.

1.3 IMMUNOSUPPRESSIVE THERAPIES

The advent of modern immunosuppressive drugs revolutionized the field of transplantation surgery by allowing the survival of allografts for much longer time frames [2, 11]. While a few decades back allografts would be rejected within a few days [4, 10], it is not uncommon today for allografts to survive for several years. With longer survival times, allo-transplantation has become more common, and patients with specific organ failures are now provided with a viable treatment option that did not exist in the past. This section will review some of the most commonly used immunosuppressive agents in chronological order of their use in the clinic.

1.4 IMMUNOSUPPRESSIVE AGENTS

1.4.1 Azathioprine

Azathioprine, introduced by Calne and colleagues, is an analog of purines (bases that are part of the DNA), and inhibits immune cell proliferation through its effects on purine metabolism in cells (reviewed in [14]). It was the first drug (chemical) that allowed for long term survival of transplants (up to several weeks) by effectively suppressing rejection responses and not being lethal over a short course of treatment [15] (reviewed in [16]). Azathioprine was commonly used in combination with steroids for immunosuppression before the introduction of calcineurin inhibitors, and even today it is commonly used in triple or quadruple therapy maintenance immunosuppression [17]. Due to its generic ability to suppress cell proliferation, azathioprine has many adverse side effects, such as the suppression of bone marrow cells leading low red blood cell and neutrophil counts.

1.4.2 Corticosteroid derivatives

Corticosteroids are naturally occurring hormones in the body with many functions, including modulating the immune system. Synthetic derivatives of naturally occurring corticosteroids that have longer half lives, greater stability and lower toxicities are some of the most potent and commonly used drugs for suppressing immune responses. At high doses these agents are known to directly cause cell lysis, but at moderate doses they can affect a range of immune cell functions by influencing the cytokine production machinery [17]. These drugs are commonly used in most immunosuppressive regimens after a transplant procedure, and are often the first

choice of medication for acute rejection episodes. Long term use of corticosteroid derivatives can lead to serious health risks, which include but are not limited to, cardiovascular problems, increased risk of infection and tumors, alterations in growth hormone production and severe bone loss.

1.4.3 Cyclosporine

Cyclosporine, introduced first by Calne and colleagues [18-19], dramatically altered the landscape of immunosuppression following solid organ transplantation by delaying rejection of allografts from several months to years. Such a significant change in graft survival times was due to the high therapeutic index (i.e. effective dosage is low and toxic dosage is high) of the drug that allows for continuous immunosuppression for long periods of time with limited side effects [17]. Cyclosporine functions by inhibiting the function of calcineurin enzymes and subsequent downstream signaling events. This inhibition leads to altered secretion of many inflammatory cytokines and increased production of immunosuppressive mediators such as TGF-β [20]. Even though cyclosporine has a high therapeutic index, it is known to have severe side effects (primarily nephrotoxicity) associated with continuous use for several years. Finally, it is unable to control chronic rejection responses, which eventually leads to graft rejection [17, 21].

1.4.4 Rapamycin

Rapamycin (rapa; also known as sirolimus or rapamune; Figure 2) was discovered in the 1970's as an antifungal agent produced by *Streptomyces hygroscopicus*. Upon the discovery of the potent immunosuppressive agent tacrolimus (a drug that is similar in structure to rapa and is

known to bind the same intracellular protein), rapa was reevaluated for its ability to suppress immune responses (reviewed in [22-23]). Rapa forms a complex with FK binding protein, and this complex affects the activity of the intracellular protein mammalian target of rapamycin (mTOR). Through this interaction, rapa affects many immune cell functions, which include suppression of cellular proliferation driven by cytokines and CD28 downstream signaling in T cells [24]. A significant advantage of rapa over other immunosuppressive drugs is its low systemic toxicity and negligible nephrotoxicity [25-26] (a significant problem with the most commonly used immunosuppressants cyclosporine and tacrolimus). Hence, rapa is now commonly used in immunosuppressive regimens, and is clinically approved for renal transplantation [25, 27-28]. Although rapa is known to have low systemic toxicity in most animal models, it has been shown to adversely affect gastrointestinal functions in dogs and cause testicular atrophy in monkeys (reviewed in [23]). Even though a few of these unwanted side effects have been reported in humans too, given its therapeutic potential rapa is currently used in the clinic.

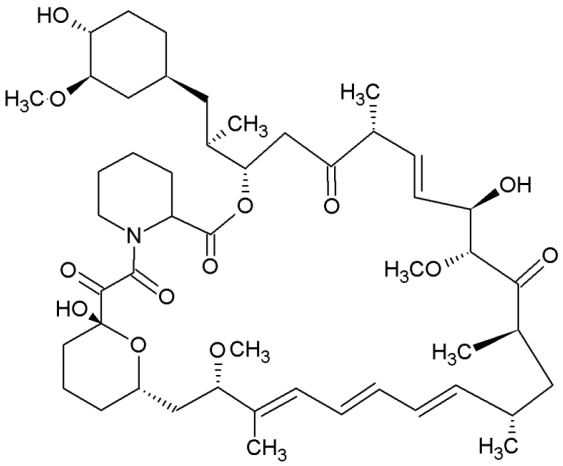

Figure 2: Structure of rapamycin

A noteworthy point about the immunosuppressive functions of rapa is its ability to alter immune cell function through mechanisms that are **independent of direct cell proliferation suppression** [29-31]. For example, rapa is known to affect DC function driving the cell towards a more "tolerogenic" phenotype, and has the potential to synergize with TGF-β to induce naïve T cell to regulatory T cell conversion [32] (discussed in greater detail in later chapters). One of the primary focuses of this dissertation will be to exploit the two aforementioned functions of rapa for the promotion of immune tolerance.

1.4.5 Immunosuppressive antibodies

Antibodies against immune cell receptors are commonly used to block the function of these cells or to deplete them through the complement or antibody dependent cell cytotoxicity pathways. Immunosuppressive regimens that include such antibodies are very useful as a prophylactic treatment before transplantation and to quickly treat acute rejection episodes (where a rapid depletion of immune cells and their functions, is required). Antilymphocyte antibodies currently in use in the clinic are mainly directed against a variety of receptors expressed on T cells. For example, anti-CD3 antibody (Muromonab®) was the first antibody approved for use in humans as a drug. It rapidly depletes all CD3 expressing T cells from circulation, thereby suppressing adaptive immune responses for a short time (reviewed in [33]). New CD3 expressing T cells arise soon after cessation of treatment with Muromonab®. Muromonab® cannot be used for long time-periods as repeated administration of this antibody leads to the development of neutralizing antibodies against anti-CD3, which reduces the efficacy of this treatment. Another antibody preparation, against CD52 (anti-CD52, developed by Waldmann and colleagues) [34-35], has been quite effective at suppressing immune responses due to its ability to deplete immune cells

expressing CD52 (T cells, B cells and NK cells among other immune cells). Currently, this antibody is used in triple and quadruple immunosuppressive regimens [36-37](reviewed in [17]). While effective, antilymphocyte antibodies can increase the risk of infections and tumors; hence, their clinical use is restricted to short treatment durations and usually only during acute rejection episodes.

1.5 TOXICITY PROBLEM

One significant disadvantage of all of the aforementioned immunosuppressive drugs is their toxicity to native cells of the body due to systemic administration. Most pharmaceutical chemicals have this problem, but the deleterious effects are greatly magnified in the case of immunosuppressive drugs due to the need for life-long administration of these agents. Given that most of these agents have similar toxicities to liver, gastrointestinal and kidney tissue, it is difficult to solve the toxicity problem just by altering the immunosuppressive drug administered. Instead, one viable alternative is to develop strategies to deliver the immunosuppressive drugs specifically to the transplant site instead of systemically.

1.6 DRUG DELIVERY APPROACHES FOR IMMUNOSUPPRESSION

1.6.1 Introduction to Drug Delivery

Today, it is well recognized that delivery of therapeutic agents at appropriate doses, precise time intervals and to specific locations in the body is necessary to observe the best possible treatment effect. Drug delivery formulations that achieve these goals are known to improve effectiveness of the therapeutic [38]. Historically, the most commonly used formulations have been pills (that are consumed orally) and liquids (that are injected intravenously). However, these formulations have two major disadvantages: (i) systemic delivery of drugs is often associated with toxicity to liver, kidney and gastrointestinal cells; and (ii) inability to maintain constant drug levels in the body leading to the presence of either toxic (high) or non-therapeutic (very low) concentrations in the blood. To solve the first problem, many different drug targeting strategies have been developed (discussed in detail in [39-40]). Although immunosuppressive drug targeting strategies hold tremendous promise, suppressing rejection responses using this approach remains difficult due to the complexity and redundancy of immune responses against a graft. Further, while it is relatively easy to actively target biological therapeutics (e.g. antibodies), targeting small molecules (e.g. immunosuppressive drugs) remains a significant challenge.

The second problem with the use of pills and injections is the inability to maintain constant drug levels in the body (Figure 2A) and the need for regular and frequent consumption of these drug formulations, which requires a high degree of patient compliance. Sustained release formulations were designed to overcome these limitations (Figure 2B) [41]. Sustained release systems are capable of providing spatial as well as temporal control over the release of the drug. In these formulations, the drug is usually combined with an inert degradable polymeric material

that can serve as a slow-release drug depot. Upon injection or implantation in the body, the drug is released from the formulation based on the degradation rate of the polymer as well as passive diffusion kinetics of the drug through the material. The amount and rate of drug release can be controlled by altering the (i) quantity of drug loaded into the material, (ii) method of loading, and (iii) properties of the material. Some of the first such formulations were fabricated close to half-a-century ago [42].

One of the most important uses of sustained release formulations is the ability to encapsulate and controllably release biological macromolecules such as proteins and DNA. Biological agents have a narrow therapeutic window and very short half lives *in vivo*. Hence, their delivery through traditional pills and injections is cumbersome and expensive. In a seminal paper in the field of drug delivery, Langer and Folkman showed for the first time that polymer based formulations can be used for sustained release of biological macromolecules [43], thereby solving many of the problems associated with using these macromolecules as drugs. Since then there have been numerous reports on the development of novel formulations for a variety of small molecules as well as biological agents (reviewed in [44] and [45]).

1.6.2 Controlled release of immunosuppressive agents

The earliest reports of sustained release formulations for immunosuppressive drugs were published two decades ago [46-48]. Subsequently, there have been multiple reports on the encapsulation and controlled release of immunosuppressive agents. For example, sustained release formulations for cyclophosphamide [46], dexamethasone [49-50], cyclosporine [47, 51-53], tacrolimus [54-56], and rapamycin [57-59] have been fabricated previously. Intra-transplant injections of these formulations can maintain high local levels of drug while reducing systemic

toxicity. However, local application of controlled release vehicles is limited by the fact that immune responses against a graft usually arise at secondary lymphoid tissues that are distal to the transplant site. Sustained release formulations can also be used as depots for controlled release of immunosuppressive drugs (that would maintain a constant drug level in the blood). However, systemic toxicity still remains an issue in this case. Further, the formulations described above cannot overcome drawbacks that are inherent to immunosuppressive drugs, which are discussed in the next section.

1.7 ALTERNATIVE APPROACHES TO IMMUNOSUPPRESSION

Direct suppression of immune cell proliferation mediated by immunosuppressive drugs is associated with many side-effects [60-61], some of which have been discussed in earlier sections of this chapter. Additionally, it is known that these agents are unable to prevent chronic rejection, and may act as a barrier to the indefinite acceptance of the transplanted tissue [62-64]. Importantly, these critical drawbacks are inherent to the immunosuppressive strategy employed by these drugs and cannot be solved by developing new drug delivery formulations (as discussed in the previous section). Thomson and colleagues [65-66], Dallman [12], Kaplan and colleagues [62] among many others have suggested that the best possible treatment for transplant rejection is getting the immune system to accept the transplanted tissue as self, a phenomenon that is termed as *tolerance*. The next few sections introduce the concepts of tolerance and describe some of the cell types that maintain immune homeostasis in the steady state and discuss their role in preventing transplant rejection.

1.8 IMMUNO-TOLERANCE AND TOLEROGENIC DENDRITIC CELLS

1.8.1 Tolerance

The mechanisms utilized by the immune system to defend the body from infectious agents and abnormal native cells are capable of damaging self-tissue. The immune system has developed elaborate means to prevent such collateral damage; either through inactivation of immune cells that act against self tissue (tolerance) or by closely regulating inflammatory responses at a disease site (immunoregulation that can involve tolerance inducing mechanisms) [66-67]. A key feature of immunological tolerance is the ability of the immune system to differentiate between self tissue and pathogens. Burnet and Fenner first postulated the presence of tolerance in the immune system - "The failure of antibody production against autologous cells demands the postulation of an active ability of the reticulo-endothelial cells to recognize 'self' pattern from 'not-self' pattern in organic material taken into their substance" [68] (adapted from Baxter [69]). Following the postulation of this theory, research has demonstrated that the immune system maintains tolerance to self tissues through many different means, which can be combined into two broad groups – central and peripheral tolerance [70].

Central tolerance is the process by which the immune system deletes T and B cells that respond to self antigen. The process of genetic recombination that gives rise to T and B cell receptors with different antigen specificities does not exclude them from recognizing self-antigen. To prevent damage to self-tissue, these T and B cells are induced to undergo apoptosis during the maturation process in the thymus and bone marrow, respectively [70]. However, not all self-reactive cells are successfully eliminated in the thymus and bone marrow through these processes [71-72].

Peripheral tolerance is the process of preventing activation and proliferation of self-reactive cells that reach lymphoid circulation. It is chiefly maintained by two distinct cell populations: (i) *dendritic cells (DC) that are "tolerogenic"* (tolerance generating); and (ii) *regulatory T cells (Treg)*. Tolerogenic DC and Treg can function together or independently. Also, each cell type is suspected to have the capability to induce the other for help in generating tolerance. The following two sections will describe tolerogenic DC and Treg, respectively.

1.9 TOLERANCE-INDUCING DENDRITIC CELLS

As discussed in Section 1.1, the initiation of adaptive immune responses is dependent on antigen presentation by DC. DC activate $CD4^+$ T cells through MHC-TCR and co-stimulatory surface receptor interactions as well as soluble cytokine based signaling. These three signaling mechanisms, together, shape the nature of the adaptive immune response from immunity against pathogens to tolerance towards self-tissue. Until the turn of the century, it was unclear how DC could control such diverse responses. Two pioneering theories (infectious-self (proposed by Janeway [6]) and the danger model (proposed by Matzinger [73-74])) helped to explain the tremendous diversity of DC function. Janeway and Medzhitov proposed that antigen presenting cells (including DC) are able to identify self from infectious agents by recognizing molecular patterns that are specifically expressed on pathogens [6, 75-76]. Matzinger proposed that immune responses are initiated when an antigen is presented to the immune system in the context of "danger signals" [73-74]. The corollary to these ideas was that in the absence of infections or danger signals, presentation of antigens by DC would be considered innocuous and tolerance

would ensue. Subsequently, these theories have been shown to hold true by many different groups (reviewed in [66, 77]).

DC are able to control immunity and induce tolerance by altering T cell activation signals. Modulation of signals that activate T cells can lead to multiple different outcomes, such as: (i) induction of T cell anergy or absence of functional activity that occurs due to MHC-antigen presentation in the absence or under very low levels of co-stimulatory signaling using CD80/83/86 [78-82]; (ii) induction of tolerance by constantly presenting antigen over long time-periods leading to T cell exhaustion (reviewed in [83]); (iii) induction of effector T cell apoptosis; and most importantly, (iv) establishment of dominant tolerance through induction and proliferation of Treg through secretion of immunosuppressive factors such as TGF-β [84-85], IL-10 [86], prostaglandins [87], or retinoic acid [88] (reviewed in [89]). The following section reviews some of the most commonly used techniques to generate tolerogenic DC.

1.9.1 Antigen presentation through immature DC

In the native homeostatic state (absence of an infection), DC exist as immature antigen presenting cells that have high phagocytic capacity while expressing low levels of co-stimulatory receptors [90-91]. As suggested in figure 1 and by Janeway [6] and Matzinger [73-74], under these conditions, DC are poor activators of T cells. This natural state of DC is used by the body to induce and maintain tolerance against self antigen. Native proteins and apoptotic cells are phagocytosed readily by immature DC. This process occurs in the absence of danger signals (apoptotic cells and native proteins do not activate the “danger receptors (TLR)” on DC), leading to native antigen presentation in the absence of co-stimulation (co-stimulatory receptors have been shown to be upregulated only upon TLR activation [92]). Such antigen presentation in the

absence of co-stimulation generally leads to T cell anergy [78-79], which allows for establishment of tolerance against the native antigen.

Immunologists have attempted to exploit this mechanism of generating tolerance for treating autoimmunity and transplant rejection. For example, Steinmann and colleagues have shown that targeted delivery of any antigen (a small peptide or an intact whole protein) to quiescent DC (using antibodies against DC receptors such as DEC-205) can induce tolerance towards that particular antigen [93-94] (reviewed in [91]). This approach has subsequently been used to suppress and prevent immune responses in murine models of diabetes [95] and experimental autoimmune encephalomyelitis [96]. Similarly for transplantation, researchers have recognized that adoptive transfer of immature allogeneic DC (DC isolated from the donor) can induce donor antigen-specific tolerance [78-79] (reviewed in [65]). Immature donor-specific DC have been shown to induce T cell anergy [78, 80] and drive Treg proliferation in vitro [97-98], as well as significantly prolong survival of heart [78] and islet allografts [99].

1.9.2 Altering DC function using genetic material

The task of reducing expression of co-stimulatory surface proteins or secretion of inflammatory cytokines, or increasing the production of immunosuppressive proteins to generate a tolerogenic DC can effectively be achieved by using silencing RNA and recombinant gene constructs respectively. For example Thomson and colleagues have suggested that transfecting DC with recombinant genes that code for IL-10, CTLA-4, or TGF-β, among many other proteins, can be a potential approach to generating tolerogenic cells [65, 100-101]. However, the potential for this approach has not been realized due to fears over the safety of the use of genetic constructs in humans and difficulties in achieving high transfection efficiencies in DC. An alternative

approach pioneered by Giannoukakis and colleagues is the use of antisense RNA to silence gene products of co-stimulatory surface proteins CD40, CD80 and CD86 [102]. Their work has lead to the initiation of clinical trials at the University of Pittsburgh Medical Center using this technology [103].

1.9.3 Modulation using immunosuppressive pharmacological agents

During the past two decades, numerous studies have enumerated the role of immunosuppressive and immunomodulatory agents in modulating DC function ex vivo and in vivo (reviewed in [31]). Corticosteroids, vitamin D_3 (and its metabolite 1α,25-dihydroxyvitamin D_3), cyclosporine, tacrolimus, mycophenolate mofetil, aspirin and rapamycin (rapa) are some of immunosuppressive drugs that have been shown to affect DC differentiation / maturation, thereby altering their function. Most of these agents negatively affect intracellular pathways responsible for up-regulation of co-stimulatory molecules and/or secretion of inflammatory cytokines, while some can positively affect immunosuppressive pathways by increasing the production of transforming growth factor β1 (TGF-β), IL-10 and indoleamine 2,3-dioxygenase (IDO). These agents can also affect the migratory capacity of DC, thereby altering their ability to interact with lymphocytes in vivo.

One immunosuppressive agent that is of special interest to transplant immunologists is rapa, as it suppresses the ability of DC to activate effector T cell proliferation, while promoting Treg proliferation [32]. Rapa inhibits downstream signaling from the kinase mammalian target of rapamycin (mTor), which leads to impaired DC maturation and T cell stimulatory function. Thomson and colleagues show that rapa treatment of *in vitro* bone marrow derived DC, decreased expression of co-stimulatory molecules on the surface as well as lowered secretion of

IL-12 and tumor necrosis factor (TNF) [30]. Additionally, they also show that rapa-treated DC can be used as a cellular therapy for the prolongation [104] and promotion of indefinite survival of heart allografts [32]. These results hold promise for the clinical translation of rapa-conditioned DC as a treatment for delaying graft rejection and potentially inducing transplant tolerance.

1.9.4 Tolerogenic DC in the clinic

Promising results obtained from clinical trials of DC-based therapies for cancer provide support to the idea that a tolerogenic DC based approach can be used to promote immuno-regulation [65, 105]. A large number of studies in small animal models (mouse and rat) have highlighted the efficacy of in vitro generated tolerogenic DC in promoting tolerance to transplants. For clinical translation of this research, an important and necessary step is to replicate the aforementioned studies (generating tolerogenic DC and testing for induction of tolerance) in large animal models (non-human primate) [65, 106]. Thomson and colleagues have been working towards generating tolerogenic DC in the Rhesus monkey [107-108], with the ultimate goal of optimizing protocols for the use of DC therapy in humans. Data from these studies will go a long way towards the translation of tolerance generating cellular therapies to the clinic. Further, along these lines, it is important to note that Giannoukakis and colleagues have started (for the first time) clinical trials to test the effect of using antisense RNA treated DC as a therapy for type-I diabetes [102-103]. This approach has the potential to be applied in promoting graft tolerance as well.

Regardless, an important aspect to consider while developing tolerogenic DC-based therapies are the problems associated with ex vivo cell culture. Isolation and culture of these cells under GMP conditions is cumbersome, expensive and often plagued with problems of stability and purity of cell populations. In vivo targeting of DC with tolerance inducing agents

has been suggested as a potential alternative to bypass these problems [65, 101]. Candidate therapies under development along these lines follow the previously described techniques to generate tolerogenic DC: (i) DC specific antigen delivery - DEC-205 based targeting of antigens to immature DC *in vivo* [91, 95]; and (ii) altering DC function with genetic material - delivery of antisense-RNA using appropriately sized microparticles to phagocytic cells in vivo [102]. Systems for the *in vivo* modulation of DC function, by targeting immunosuppressive drugs to these cells, are yet to be developed. Given the relative safety (approved by the United States Food and Drug Administration) and diversity of immunosuppressive drugs, such targeted therapeutics might dramatically reduce the time to develop a therapy that can be used in the clinic.

1.10 REGULATORY T CELLS AND IMMUNOREGULATION

1.10.1 Historical perspective:

Over the past couple of decades, immunologists have identified that not all self-reactive T cells are deleted in the thymus and that many of them are present in the lymphoid circulation [71-72]. The presence of self-reactive T cells in the periphery posed an interesting conundrum – why don't these cells initiate immune responses against self in healthy individuals? Pioneering studies in the 1980's and 90's demonstrated that the immune system has active suppressive mechanisms in place to prevent self-reactivity, and that this suppression is mediated by "certain" T cells [109-111] (reviewed in [112]). For a long time, the presence of such suppressive T cells was viewed with skepticism, mainly due to difficulties in identifying a standard set of specific markers that

allowed for isolation and study of these cells. For example, the Mason group used CD45 isoforms to distinguish the suppressor T cells [111-112], while the Sakaguchi group used CD5 [110]. But neither of these markers could be used to distinguish suppressive cells from effector cells across species. Further Weiner and colleagues described a TGF-β secreting suppressive Th3 type cell that could be induced in the periphery upon oral antigen feeding, but these cells expressed no specific markers that distinguished them from other T cells [113]. Additionally, Roncarlo and colleagues identified a separate type of suppressor T cell that could be induced after antigenic stimulation in the presence of IL-10 [86], which was later labeled as Tr1 cells. Acceptance of the existence of suppressive T cells (also termed as regulatory T cells) and extensive studies on them began following the identification of the CD25 surface protein as a discerning marker for regulatory T cells in 1995 by Sakaguchi and colleagues [114] (CD25 is expressed on activated effector T cells as well, but in a naïve uninfected state, CD25 expressing cells are predominantly suppressive). Since this landmark paper, thousands of scientific manuscripts have been published on regulatory T cells and these cells are now considered one of the central components of the mammalian immune system. Following the identification of CD4 and CD25 expressing cells as regulatory T cells, multiple other types of regulatory cells have been described [115-117]. This dissertation will only focus on the FoxP3 expressing $CD4^+$ $CD25^+$ T cells, which are termed as "Treg" (acronym for regulatory T cells) henceforth.

1.10.2 Regulatory T cell phenotype:

Following the identification of CD25, researchers have been able to demonstrate that Treg express many other surface receptors at levels that are higher than naïve or effector T cells [118-122]. Some of the most important of these receptors are: CTLA-4 (cytotoxic T lymphocyte

antigen-4) – a surface receptor that binds to the co-stimulatory (signal 2) receptors on APC allowing it to influence DC and T cell behavior; GITR (glucocorticoid-induced tumor necrosis factor receptor-related protein) – suggested to play an important role in Treg suppressive function although the specific details are unclear; and FR4 (folate receptor 4) – a surface protein that is required for folic acid sensing and uptake, which in turn prolongs Treg survival. In addition to these proteins, Treg also express adhesion proteins (e.g. CD62L, CD103) and chemokine receptors (e.g. CCR7, CCR4, CCR5 e.t.c.) at high levels depending on state of activation and the need to migrate to specific tissues. It is noteworthy to mention that none of these receptors are unique to Treg, and are expressed by naïve or effector T cells, but generally at lower levels. To date, no surface protein has been identified that distinguishes Treg from other T cells.

In 2003, three independent groups identified that Treg exclusively express the intracellular transcription factor FoxP3 (also known as scurfin) [123-125]. These studies provided evidence that FoxP3 expression determined both Treg phenotype and function. More specifically, deletion of the FoxP3 gene led to a failure in Treg development and a corresponding absence of suppressive function. Additionally, induction of FoxP3 in naïve T cells resulted in corresponding induction of suppressive function in those cells. Further, depletion of Treg from normal mice through blocking antibodies caused autoimmune disease. Finally, transfer of these cells from wild type mice could mitigate autoimmune disease and restore immune homeostasis (reviewed in [126]).

FoxP3-expressing Treg can further be divided into two specific subtypes: (i) natural Treg (nTreg) that arise in the thymus are also thought to express TCR specific for self antigen; (ii) peripherally induced Treg (or adaptive Treg, iTreg) that arise in the periphery. nTreg have been

described to be anergic under standard *in vitro* conditions [122], but are capable of robust *in vivo* proliferation. They require high levels of IL-2, TGF-β and possibly folic acid, among other factors, for their survival and proliferation [126]. iTreg are generated *in vitro* and *in vivo* upon T cell activation in the presence of TGF-β. iTreg can develop upon antigenic stimulation in an immunosuppressive environment rich in TGF-β *in vitro* [84-85], and in the presence of TGF-β and retinoic acid *in vivo* [88]. Another possible mechanism for their *in vivo* generation is their induction in the presence of nTreg, a phenomenon that is described as infectious tolerance [127]. FoxP3 expressing iTreg appear to function in a similar fashion to nTreg, but with an antigen recognition repertoire that is more diverse (against microbial antigens, allo-antigens, tumor antigens and possibly even self-antigen) [115]. Additionally, the long-term *in vitro* and *in vivo* stability (FoxP3 expression as well as suppressive capabilities) of iTreg remains questionable [128].

1.10.3 Treg function:

The main function of Treg is to restore immune homeostasis by suppressing effector immune cells. Treg carry out their suppressive function using many different mechanisms (reviewed in [127, 129]), which can be broadly categorized as contact-dependent and contact-independent methods.

1.10.3.1 Contact-dependent suppression

(i) *Treg suppression mediated through interactions with DC* – Treg express CTLA-4 at high levels, and interactions with DC through CTLA-4 can lead to up-regulation of the immuno-modulatory protein indoleamine 2, 3-dioxygenase or down-regulation of surface co-stimulatory

molecule expression on DC. Both of these conditions lead to an inability to activate T cells effectively and have the potential to induce T cell apoptosis [126, 130]. Alternately, Treg can interact with DC to prevent their proliferation or even directly lead to their apoptosis, as suggested by studies on complete removal of Treg from adult mice that show a marked increase in the numbers of DC in these animals[131]. Additionally, Treg might be able to simply outcompete T cells in their ability to interact with DC and prevent naïve T cell – DC interaction (suggested in [126]).

(ii) *Treg suppression mediated through interactions with T cells* – Many activated Treg have been shown to express Granzyme B and Perforins on their surface, which can promote T cell apoptosis following Treg-T cell interaction [132-133]. It has also been suggested that upon on direct interaction, Treg and T cells form gap junctions through which Treg can deliver immunosuppressive molecules to T cells [134] or physically prevent them from proliferating [126].

1.10.3.2 Contact-independent suppression:

(i) *Active Treg suppression mediated through release of immunosuppressive cytokines* – Depending on the nature of inflammation, the tissue environment and the activation state, Treg are known to release a number of different immunosuppressive mediators that can actively modulate immune cell function. One of the most important factors is TGF-β, which has been suggested to be not only a suppressive mediator but also essential for Treg survival. IL-10 and adenosine are other factors that Treg secrete or use to modulate T cell responses [86, 134]. The secretion of these modulators causes a decrease in immune cell proliferation and generally suppresses inflammatory cytokine production [86, 135-136], both of which are essential for re-

establishing homeostasis. Additionally, the secretion of immunosuppressive factors results in one of the most widely described modes of Treg suppression – "*bystander suppression*" [127]. In this type of suppression, Treg not only suppress antigen-specific responses, but also suppress antigen non-specific responses due to the establishment of an immunosuppressive environment. This mechanism allows Treg to directly control the suppression of many other immune cells such as CD8+ T cells, B cells, macrophages, and NK cells [137]. Further, using these mediators (especially TGF-β), Treg can induce naïve as well as effector T cells to become suppressive Treg cells, a phenomenon called "*infectious tolerance*" [126-127].

1.10.4 Regulatory T cell therapies in the clinic

Given their unique capability in regulating the immune system, regulatory T cells are considered as one of the best candidates for tolerance inducing therapies for graft rejection as well as autoimmunity [126, 138-139]. Indeed, a significant amount of research has gone into developing Treg based cellular therapies for use in humans (reviewed in [138-139]). Recently, clinical trials on using Treg to treat graft versus host disease (GVHD) have begun in Germany (Edinger and colleagues) and the US (Blazar and colleagues) [138], and it is expected that such clinical testing will expand to other types of transplants as well as autoimmune diseases [138]. However, for the widespread clinical translation of Treg therapies, three major hurdles need to be cleared: (i) *isolating Treg specifically in GMP conditions* – as mentioned in previous sections, a surface marker for Treg has not been identified, which makes it difficult to isolate pure populations of Treg [138-140]; (ii) *increasing Treg numbers ex vivo* – given the small number of cells that can be isolated from the peripheral blood in humans, it is necessary to proliferate Treg ex vivo prior to adoptive transfer. Unfortunately, Treg do not proliferate efficiently under in vitro activating

conditions [122, 126, 140]; (iii*) ensuring that Treg don't lose their functional suppressive capacity in vivo following adoptive transfer* – the single greatest fear associated with Treg cellular therapies is the loss of their suppressive capability and reversion to a effector T cell phenotype after adoptive transfer [138, 140].

Although there have been noteworthy developments in overcoming these challenges in the recent past (e.g. improved methods for GMP isolation of Treg [141-142] and *ex vivo* Treg expansion [143-145]), significant problems associated with Treg stability and the expense of Treg cell therapy still remain. Hence, alternatives to Treg cellular therapy are actively being sought. An interesting option would be to develop strategies that increase local numbers of Treg *in vivo* at the site of a transplant. Currently, there are no known strategies to manipulate Treg cells in vivo. The following chapters focus on the development of the first synthetic strategies to increase in situ numbers of Treg.

2.0 DELIVERY OF RAPAMYCIN TO DENDRITIC CELLS USING DEGRADABLE MICROPARTICLES

2.1 INTRODUCTION:

As highlighted in Chapter 1, graft rejection can be delayed and possibly prevented through the generation of tolerogenic DC that specifically suppress immune responses against the transplant. One potential method of generating tolerogenic DC is by using immunosuppressive drugs, such as rapa, to modulate DC function [30-31]. However, the problem of DC-specific delivery of rapa needs to be addressed, as systemic delivery of this drug is associated with adverse side effects [146-147].

DC-specific delivery of therapeutic agent like rapa can be achieved through passive targeting, in which, the therapeutic is encapsulated into polymeric or lipid based vesicles (such as microparticles) that can specifically be taken up by phagocytic cells and are too large for non-phagocytic cells to envelop (0.5-5 μm) [148]. The size of the microparticle is an important aspect to consider while developing DC-specific delivery vehicles, as: 1) if the particles are too small (nanoparticles) they will be taken up by many different cells of the body, 2) if they are too big (>10 μm) they will not be taken up by DC. We and others have previously shown that microparticles within the size range of ~1-10 μm are specifically taken up by phagocytic APC, such as DC [148-150]. Furthermore, it has been suggested that such microparticle-based delivery

vehicles can achieve markedly higher potency with orders of magnitude less drug as compared to systemic delivery or even local delivery, due to the capability for targeted and intracellular delivery of payload to phagocytes [151].

Given this information, we hypothesized that intracellular delivery of rapa to DC using microparticles loaded with the drug would be better at inducing a tolerogenic phenotype when compared to treatment of DC with soluble rapa *in vitro*. In this chapter, we describe the development and characterization of an appropriately sized (1-10 μm diameter) microparticulate system for DC-specific intracellular delivery of rapa, to test the aforementioned hypothesis.

2.2 METHODS:

2.2.1 Microparticle Preparation

Microparticles were prepared using the single emulsion/evaporation technique as described [152-153]. Briefly, 200 mg poly(lactic-co-glycolic)acid (RG504H – viscosity 0.45-0.6 dl/g, Boehringer Ingelheim Chemicals Inc., Petersburg, VA) was dissolved in 4 ml dichloromethane (oil phase). One hundred μl of a stock solution of rapamycin (LC laboratories, Woburn, MA) in Dimethyl Sulfoxide (10mg/ml) was dissolved in the oil phase, which was then homogenized at 10100 rpm for 1 minute in a 2% PVA (MW ~ 25,000, 98% hydrolyzed; Polysciences) solution, using a homogenizer (Silverson L4RT-A). This emulsion was immediately poured into 90 ml of 1% PVA solution, and dichloromethane was allowed to evaporate. After 3 hours, the particles were centrifuged (1500g, 10 min, 4°C) and washed x4 in deionized water. Microparticles were then re-suspended in 5 ml of deionized water, frozen on dry ice and lyophilized (Virtis Benchtop

K freeze dryer, Gardiner, NY; operating at 60 mTorr). Fluorescently labeled rapamycin-microparticles were prepared by adding 100 µl of 2mg/ml (in DMSO) Alexa Fluor 647 carboxylic acid, succinimidyl ester (Invitrogen) to the oil phase along with rapamycin, and following the same protocol as above.

2.2.2 Microparticle Characteristics

Microparticles were sized and counted using volume impedance measurements on a Beckman Coulter Counter (Multisizer 3). Average size was determined by counting at least 10,000 particles. Microparticle surface morphology and shape were examined using a scanning electron microscope (JEOL JSM-6330F, Peabody, MA). The surface charge of microparticles was determined by zeta potential measurements (ZetaPALS, Brookhaven Instruments).

2.2.3 Measuring encapsulation efficiency

Five mg of rapamycin-microparticles (rapaMP) were dissolved in 1 ml of acetonitrile (HPLC grade), sonicated for 5 min and left under constant vortex for 30 min. The amount of rapamycin was then determined by measuring absorbance of this solution at 278 nm using a UV-equipped plate reader (SpectraMax M5, Molecular Devices). Encapsulation efficiency was calculated as the ratio of rapamycin present inside particles to the amount of rapamycin that was initially added during microparticle preparation; and normalized based on the yield.

2.2.4 *In vitro* release studies

The *in vitro* release profile of rapamycin from rapaMPs was measured as described. A known amount of rapaMP was suspended in a solution of 1ml of phosphate buffered saline (DPBS, pH 7.4, GIBCO, Invitrogen), or 1 ml of 100mM phosphate buffer (pH 5) containing 0.2% Tween-80. These suspensions were placed on an end-to-end rotator, at 37 °C. At regular intervals, the particles were centrifuged, the supernatant collected, and the particles re-suspended in equal volume of the buffer. Rapamycin content in the supernatant was analyzed by measuring absorbance at 278 nm as described above. Tween-80 was added to maintain sink conditions, and prevent adhesion of rapamycin to the sides of the tubes used for measuring release.

2.2.5 Dendritic cell (DC) cultures and exposure to MP

Bone marrow-derived myeloid DC were propagated from C57BL/10 (B10) mice (The Jackson Laboratory, Bar Harbor, ME) and purified as described [154]. Briefly, single cell suspensions of B10 bone marrow were cultured for 7 days with 1000 Units/ml of GM-CSF and IL-4 in complete media supplemented with 10% FCS. On day 8 non-adherent cells were collected and CD11c+ cells positively selected using anti-CD11c-cojugated Miltenyi beads (Myltenyi Biotech Auburn, CA). Where indicated, blankMPs or rapaMPs were added (0.11 mg of rapaMPs to achieve a 5:1 ratio of particles to DC) to the culture the day before harvesting, and the culture left undisturbed till subsequent experimentation. DCs exposed to 10 ng/ml soluble rapamycin (Sigma-Aldrich) were used as additional comparison group.

2.2.6 Live cell imaging

One day after the addition of rapaMPs, DC were purified as previously indicated, and cultured for 2 hours over glass bottom culture dishes (MatTek Corporation, Ashland, MA). Adherent DC were then imaged using differential interference contrast microscopy (inverted Olympus 1X81).

2.2.7 Flow cytometric analysis:

Following incubation with blankMP or rapaMPs for either 1 day or 4 days, DC were collected from the cultures and viability determined by Annexin-V/7-aminoactinomycin D (7-AAD) staining. Collected cells were stained with FITC-conjugated anti-CD11c mAb (BD PharMingen), PE-conjugated AnnexinV and 7-AAD (Apoptosis Detection Kit, BD PharMingen), following the manufacturer's instructions and then analyzed with a LSR-II flow cytometer (BD Bioscience).

To determine the level of expression of cell surface stimulatory molecules, DC incubated with fluorescent-rapaMPs for 1 day were collected and stained with FITC-conjugated anti-CD11c mAb, PE-conjugated anti-MHC-II, anti-CD86, or anti-CD40 (all from BD PharMingen), following manufacturer's instructions and analyzed as above. For both the experiments FITC-conjugated anti-CD11c mAb was used to gate for DC using flow cytometry.

2.2.8 T cell isolation

$CD4^+$ T cells were purified from spleen and lymph node cell suspensions by negative selection. Non-$CD4^+$ cells were labeled with anti-CD11b, anti-TER-119, anti-Gr-1, anti-I-A/I-E, anti-$CD8\alpha$, anti-B220, and anti-Gr-1 mAbs (all from BD Pharmingen). Following incubation with

anti-rat-IgG-Dynabeads (Dynal Biotech, Invitrogen), bead-bound cells were removed by magnetic isolation. Purity of $CD4^+$ T cell preparation was assessed by flow cytometry and corresponded to 90%, reproducibly.

2.2.9 Mixed leukocyte reaction (MLR)

B10 DC (15,000), cultured for 1 day with rapaMPs (using different ratios of rapaMPs to DC), were mixed with 150,000 CD4 T cells (isolated from lymph nodes and spleen of normal BALB/c mice) in u-bottomed 96 well culture plates. The cells were co-cultured in complete RPMI 1640 (GIBCO, Carlsbad, CA), supplemented with 10% fetal calf serum, for 3 days following which tritiated thymidine was added. After 16 hrs, the amount of thymidine incorporated into the cells was measured using a Wallac 1205 Betaplate analyzer. A no treatment (NT) group consisting of DC that were not exposed to any particles, and DC cultured with blank particles (5:1 – particle to DC ratio) served as a negative controls. A DC group treated for 1 day with soluble rapamycin (10ng/ml) acted as a comparative control.

2.2.10 Statistics

Statistical analysis was performed using the 2-tailed Student's t-test, and all results are expressed as ± 1 SD. Standard deviation was calculated based on n=3 experiments, unless otherwise stated.

2.3 RESULTS

2.3.1 Microparticle characteristics

Rapamycin-encapsulated PLGA microparticles (rapaMP) were prepared successfully using a single emulsion/evaporation technique. These particles were sized to be 3.4 ± 1.65 μm (diameter) using volume impedance measurements on a Beckman Coulter Counter. SEM images of these particles (Figure 3) show smooth surface morphology and confirm the size obtained from volume impedance measurements. The size distribution observed in the SEM images is characteristic of the microparticle synthesis procedure described above, and has been reported elsewhere [152, 155-156]. Further, the zeta potential of these rapaMPs was determined to be -35.38 ± 4.49 mV (n=4 particle sets).

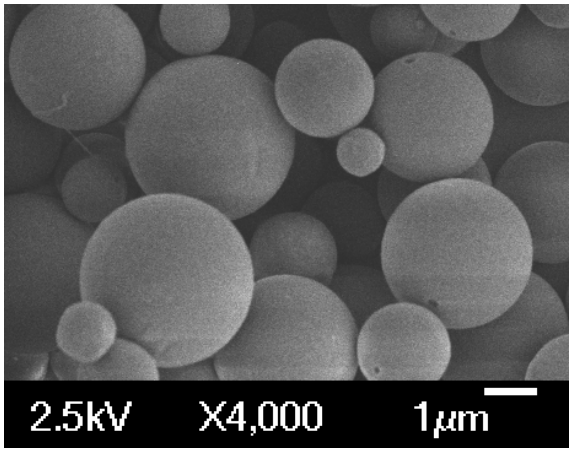

Figure 3. Scanning electron micrographs of rapamycin containing PLGA microparticles.

Representative images confirm that the particles exhibit surface integrity. Also, sizes are consistent with measurements via volume impedance (Avg. Vol. Dia – 3.4 μm).

2.3.2 Encapsulation efficiency

Encapsulation efficiency of rapaMP was determined to be 73.95 ± 5.43%. The presence of rapamycin inside microparticles was further confirmed by tandem mass spectroscopy analysis (data not shown). Based on the volume impedance measurements and encapsulation efficiencies, the amount of rapamycin in each rapaMP was calculated to be approximately 50 femtograms.

2.3.3 *In vitro* release profile

The release rate of rapamycin from rapaMPs was studied in both pH 7.4 and pH 5 buffer. It has been reported previously that DC efficiently phagocytose PLGA microparticles (3-4 μm in size) in 4-5 hrs, and that the particles may remain inside phagosomes (pH ~ 5) [149, 157]. Hence, to replicate intra-phagosomal pH conditions *in vitro,* release was studied in a pH 5 buffer. There were no significant differences observed in the release rates between pH 7.4 and pH 5 buffers (Figure 4). The release appeared to follow Fickian diffusion kinetics, and the calculated values of diffusivity of the drug in the polymer matrix were found to be in the same order of magnitude, as predicted by theoretical correlations developed by us and others [158-160]. The pH of the supernatant was measured to be stable throughout the release study (data not shown).

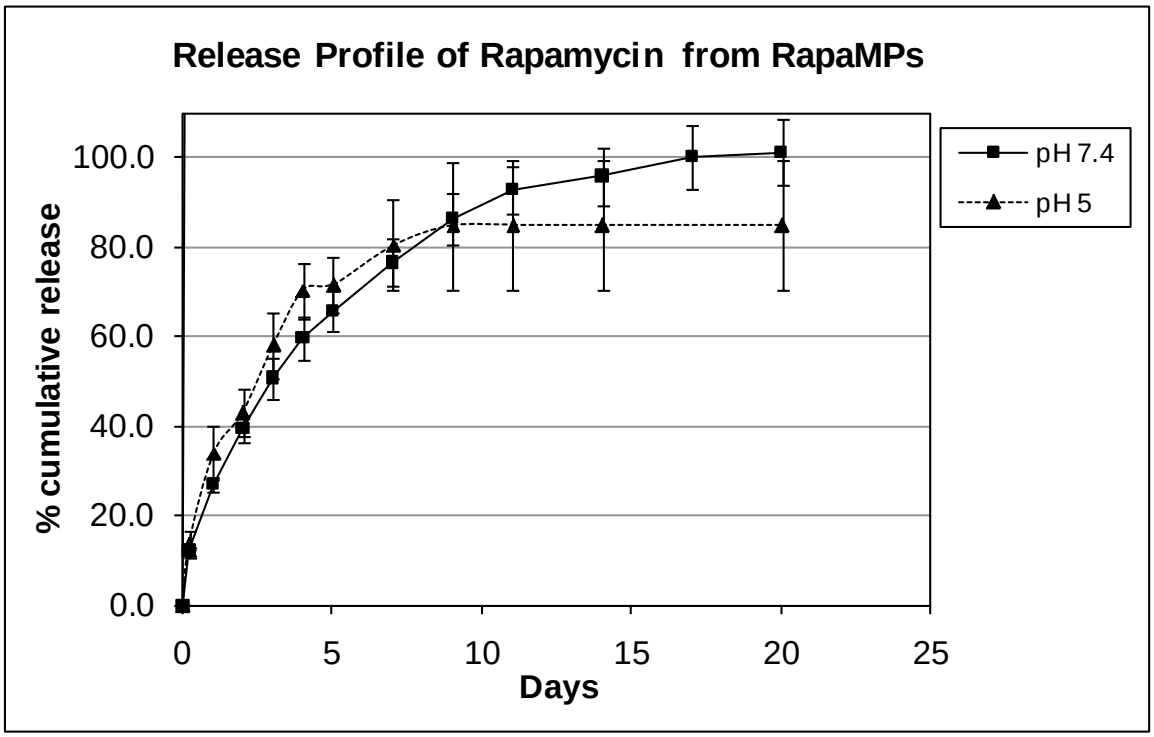

Figure 4: In vitro controlled release of rapamycin from small-rapaMPs in pH 5 and pH 7.4 buffer. Percentages are based on amount encapsulated. 100% indicates ~3.7 µg of rapamycin/mg of rapaMPs – the maximum amount that would be released if the particles degraded completely. Standard deviations are based on n = 6 samples for release at pH 7.4, and n=3 samples for release at pH 5.

2.3.4 Live cell imaging and apoptosis level

Reports in the literature have previous demonstrated that PLGA microparticles are phagocytosed by DC and can be identified inside these cells [149, 161]. Figure 5 shows images of live DC that were exposed overnight to different amounts of rapaMPs. These images show that, for any given ratio (number of particles: number of DC, adopted during the co-culture phase), a certain degree of variability in the number of particles phagocytosed by individual DC was evident. However, a general direct correlation appeared between the co-culture ratio and the average number of

particles internalized. Additionally, we observed that when a particle:DC ratio of 5:1 or 10:1 was used, the majority of DCs internalized at least one particle.

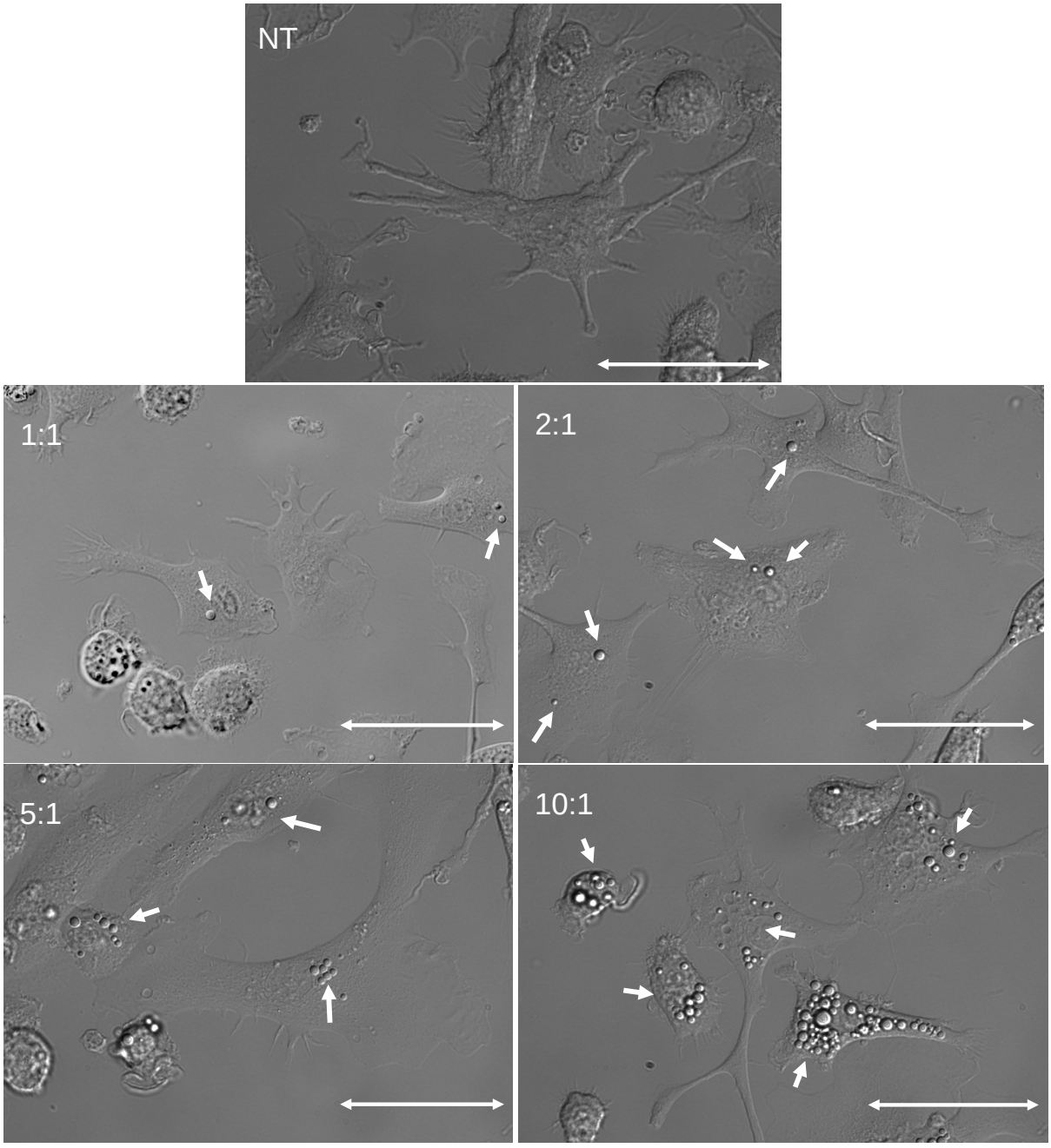

Figure 5: Representative images of live DC cultured for 1 day with rapaMPs.

NT (no treatment) implies that no particles were added to these DC cultures. The ratios indicate the number of particles added to the number of DC. Scale = 50 μm

Next, it was determined if the phagocytosis of blankMPs or rapaMPs leads to DC death. Initial trypan blue exclusion studies on cultures treated with different particle:DC ratios showed a small percentage of DC positive for trypan blue. This number was not significantly different from the control population of non-treated DC at any ratio tested (data not shown). To further investigate the status of DC exposed to MPs, we collected cells after 24 hours or 96 hours of co-incubation (particles and DC) and stained them with FITC-conjugated anti-CD11c mAb, Annexin-V (AnnV) and 7-aminoactinomycin D (7-AAD). FITC-conjugated anti-CD11c mAb was used to gate DC using flow cytometry, as CD11c is a DC-restricted marker. The combination of the other two dyes allowed the discrimination of viable DC ($AnnV^-$ $7\text{-}AAD^-$) from those that were either entering apoptosis ($AnnV^+$ $7\text{-}AAD^-$), in late apoptosis ($AnnV^+$ $7\text{-}AAD^+$), or necrotic ($AnnV^-$ $7\text{-}AAD^+$). As observed from the AnnV/7-AAD profile of $CD11c^+$ cells, 1 day after co-incubation (Figure 6A and Table 1) the proportion of apoptotic cells (early and late) among rapaMP-loaded DC was not different from that of untreated and blankMP-loaded DC. After 4 days of co-incubation (Figure 6B and Table 1), the apoptotic level of untreated or blankMP-loaded DC was not significantly different. This indicated that the process of MP phagocytosis per se was not harmful to the cells. In the rapaMP group, however, a significant increase in the proportion of early apoptotic cells was evident in comparison to untreated and blankMP groups. As blankMP phagocytosis did not induce such alteration, the pro-apoptotic effect observed is attributable to intracellular release of rapamycin.

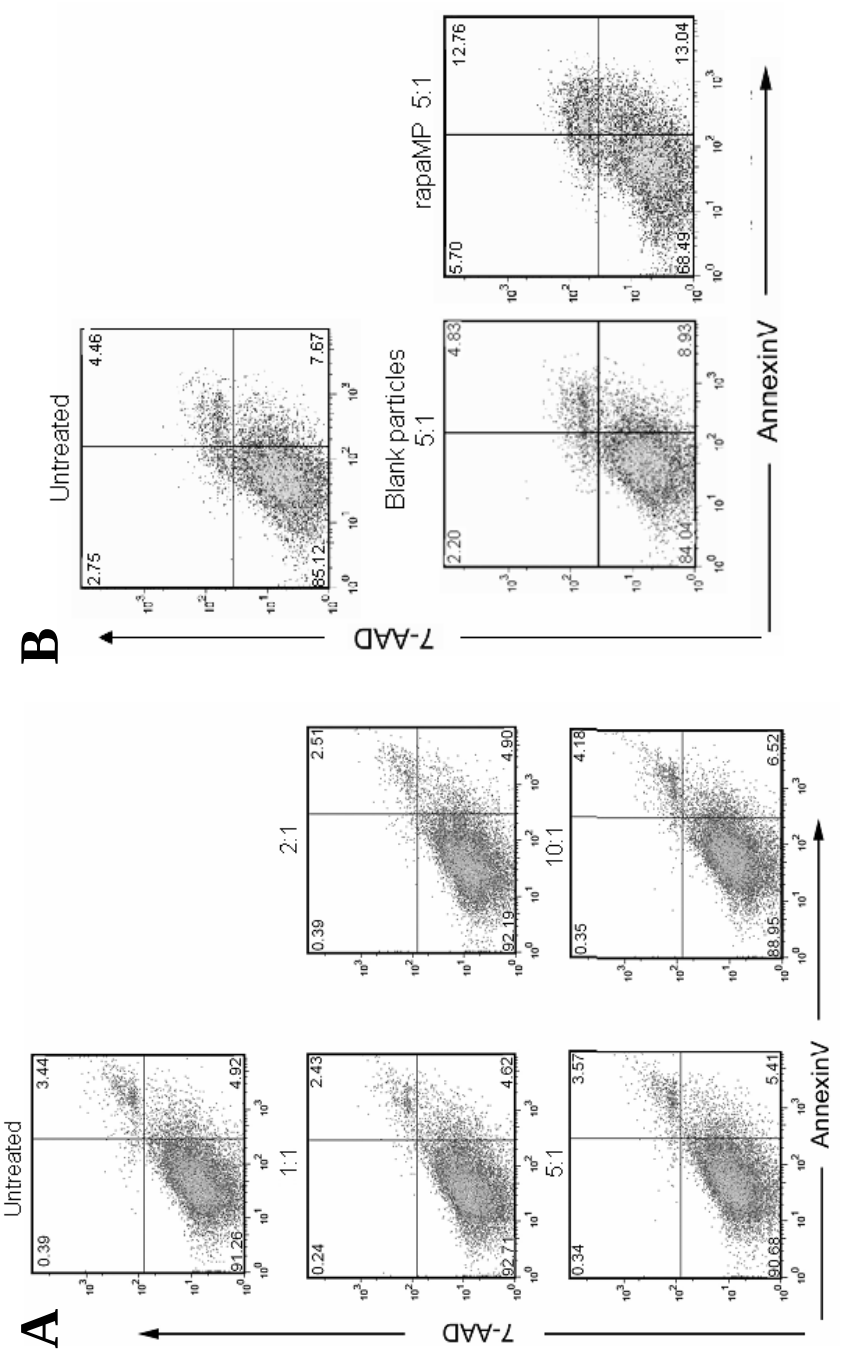

Figure 6: Annexin-V and 7-AAD staining of DC to examine potential toxicity of rapaMPs.

Apoptosis of DC incubated for (A) - 1 day, or (B) – 4 days with different number of rapaMPs was compared to untreated DC. Flow diagrams were generated by gating on $CD11c^{+}$ (DC restricted marker) cells.

Table 1: Quantitative analysis of apoptosis levels in DC that have phagocytosed rapaMPs.

Live cells represent % cells that are $AnnV^-$ $7\text{-}AAD^-$, early apoptotic represents % cells that are $AnnV^+$ $7\text{-}AAD^-$, late apoptotic represents % cells that are $AnnV^+$ $7\text{-}AAD^+$, and necrotic represents % cells that are $AnnV^-$ $7\text{-}AAD^+$. Both rapaMP and blank MP were added at a ratio of 5:1 (particle to DC). SD are based on n=3 experiments. * indicates comparison between rapaMP (4 day) and NT (4 day) groups - $p<0.05$, and ** indicates comparison between rapaMP (4 day) and blank MP (4 day) groups – $p<0.05$.

	Live cells	Early apoptotic	Late apoptotic	Necrotic
NT (1 day)	91.1 ± 1.6	5.2 ± 0.3	3.2 ± 1.0	0.5 ± 0.5
Blank MP (1 day)	89.2 ± 6.0	6.2 ± 2.4	3.7 ± 2.8	0.8 ± 0.8
rapaMP (1 day)	87.2 ± 7.2	7.4 ± 3.4	4.0 ± 3.1	1.3 ± 1.4
NT (4 day)	84.8 ± 0.5	8.2 ± 0.7	5.2 ± 0.1	1.9 ± 1.2
Blank MP (4 day)	83.7 ± 1.1	8.5 ± 0.6	6.0 ± 1.4	1.8 ± 0.4
rapaMP (4 day)	74.3 ± 7.0	12.2 ± 1.0$^{*, **}$	10.3 ± 3.1	3.3 ± 2.9

2.3.5 RapaMP phagocytosis alters DC functionality

The functional modulation of DC by rapaMPs was characterized *in vitro* using a mixed leukocyte reaction in which DC from one mouse strain (B10) were treated overnight with different ratios of rapaMP:DC and then co-cultured with $CD4^+$ T cells isolated from secondary lymphoid organs of a different mouse strain (BALB/c). T cell proliferation (an indicator of DC stimulatory capacity) was assessed by cellular incorporation of radioactive thymidine after 3 days of co-culture (a total of 4 days after addition of rapaMPs to the DCs). As shown in figure 7, a reduction in T cell proliferation that correlated with the rapaMPs:DC ratio used, was observed. Over 5 times reduction in the ability of rapaMP-loaded DC (ratio of 5:1 and 10:1) to induce T cell

proliferation ($p<0.005$) was observed when compared to blank particle-loaded or non-treated DC. Further, figure 7 shows that rapaMP treatment (at 5:1 and 10:1 ratio) resulted in significantly greater inhibition of T cell stimulatory function (about 2.5 times greater reduction in T cell proliferation) than that achieved with soluble rapamycin-treated DC ($p<0.01$). Based on the encapsulation efficiency and assuming that the rate of drug release is accurately represented by the profile described in figure 4, we calculate that the amount of rapamycin delivered to DCs by rapaMP is approximately 3 ng/ml/day, while the amount added in the soluble rapamycin-treated DC group was 10 ng/ml.

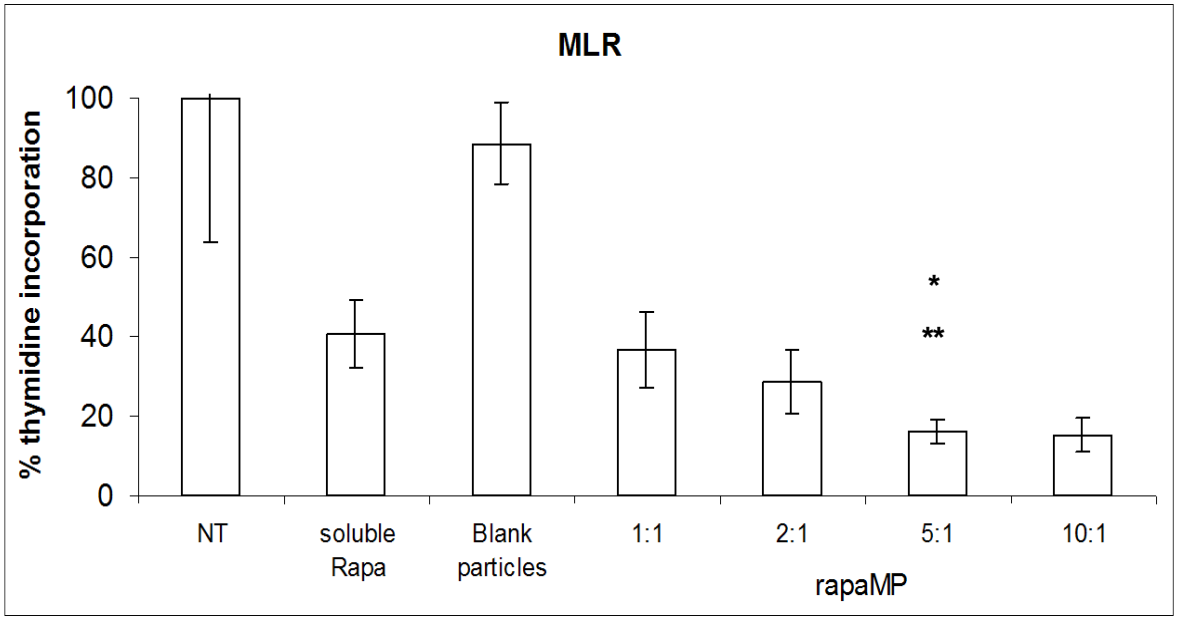

Figure 7: A mixed leukocyte reaction indicating the amount of radioactive thymidine incorporated into responding T lymphocytes.

DC were loaded with rapaMPs using the indicated MP:cell ratio and then used to stimulate allo-reactive $CD4^+$ T cells. NT (no treatment) group was assigned the value of 100% and all other percentages are normalized based on this value. The ratio of MPs to DC in the blank particles group is 5:1. Standard deviations are based on n = 6 experiments per group. * indicates comparison between 5:1 and NT groups - $p < 0.005$; ** indicates comparison between 5:1 and soluble Rapa treatment groups - $p < 0.01$.

2.3.6 RapaMP's lower expression of cell-surface stimulatory molecules

To investigate the mechanism behind functional modulation of DC loaded with rapaMPs, the expression of cell-surface co-stimulatory molecules (activation markers) was examined. Particles were loaded with Alexa Fluor 647 (A647) to track percentage of DC that have taken up particles and also determine the co-localization of particle uptake with any differences in cellular phenotype. Flow cytometric analysis revealed that DC that had taken up fluorescently stained rapaMPs (A647 high) had lowered expression of CD86, MHC-II and CD40 (common stimulatory molecules expressed on DC) when compared to DC that were A647 low (Figure 8).

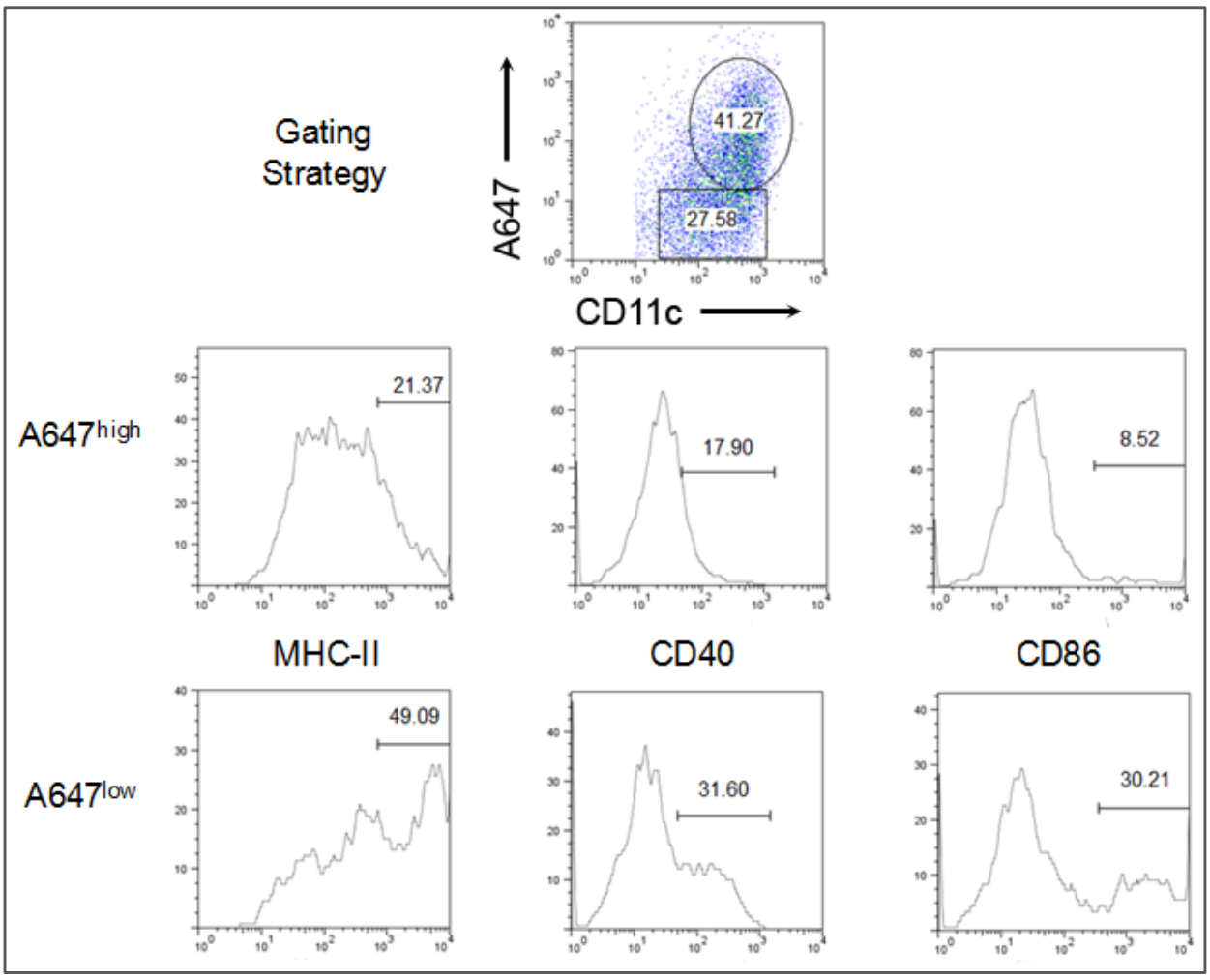

Figure 8: Flow Cytometric Analysis of DC activation markers on cells that have taken up fluorescently labeled rapaMPs (A647+).

Flow diagrams were generated by gating on $CD11c^{+}$ and Alexa-fluor 647^{high} or Alexa-fluor 647^{low}, as described in the top-center plot. Numbers on flow diagrams indicate mean fluorescence intensity values. DC were cultured with rapaMPs at a ratio of 5:1 (rapaMPs to DC) for 24 hours, prior to flow analysis.

2.4 DISCUSSION

Microparticles composed of the degradable polymer PLGA have been widely used for controlled release of drugs and proteins [44-45]. Appropriately-sized microparticles have also been used specifically for targeted delivery of drugs, antigens and DNA to phagocytic cells [148-149, 157, 161-162]. The size of the polymeric particles has an important bearing on their ability for long-term release, total payload delivered and specific targeting to phagocytic cells. If the size is too small, as is the case with nanoparticles (~200 nm or lower), it is widely believed that they are not suited to act as long term delivery vehicles [148, 163-164]. Furthermore, nanoparticles are typically taken up by all cells capable of endocytosis [148], and although such systems may deliver drugs to dendritic cells if placed in a physiologic milieu with large numbers of resident dendritic cells (e.g. the dermis) [57-58, 165] they have no inherent targeting capabilities per se. Conversely, if particles are too large (>10 μm), they cannot be internalized by phagocytic cells [166-167]. Hence, for applications where long term and phagocytic cell-specific targeted delivery is desirable (such as transplantation and autoimmune disease immunotherapy, or anti-tumor immunity) it is imperative that drugs are encapsulated into micron-sized particles (~ 1-10 μm).

One of the recently developed approaches in transplantation and autoimmune disease immunotherapy involves use of dendritic cell (DC)-based therapeutics [65, 168]. Previous reports in the literature have shown that DC cultured with specific agents, such as the immunosuppressive drug rapamycin, have altered function and can be used to suppress transplant rejection and promote donor-specific tolerance [30, 32, 104]. Our long term goal was to create a system capable of specifically targeting DC in vivo and then sustaining intracellular delivery of rapamycin to produce a more pronounced effect with respect to suppression of

transplant rejection and promotion of tolerance without systemic immunosuppression. In this study, the hypothesis that intracellular delivery of rapamycin to DC using appropriately sized microparticulates is effective at inhibiting an important initial step in the onset of transplant rejection - T cell activation, was tested.

The aforementioned experiments demonstrate that rapamycin can be encapsulated and controllably released from appropriately sized PLGA microparticles. PLGA was the polymer of choice as it does not activate DC [149], and allows for intracellular release of drug over extended periods as shown in figure 4. A Fickian release that lasts for about 3 weeks was observed, which is similar to a previously reported release profile for cyclosporine (a drug with similar mol. Wt. and hydrophobicity as rapamycin) [152]. Further, the release can be modulated as desired by changing the molecular weight of PLGA used to make the microparticles. Additionally, the data suggests that rapaMPs are associated with a more pronounced effect on DC when compared to conventional rapamycin treatments. Previously, our collaborators have shown that DC treated with soluble rapamycin are characterized by a lower capacity to activate T cells [30, 32]. However, in comparison to soluble rapamycin-treated DC, rapaMP-loaded DC exhibit significantly higher capacity in preventing T cell activation (Figure 8). Annexin V and 7AAD staining was used to analyze if this reduction was due to an increase in DC apopotosis in the presence of rapaMPs over the 96 hour period (Figure 6 and Table 3). Figure 4A shows that annexin V and 7AAD profiles for untreated and rapaMP-loaded DC following 1 day of co-incubation are similar, confirming that phagocytosis of rapaMPs does not cause increase in DC apoptosis. After 4 days of co-incubation, a small increase in apoptosis of rapaMP-loaded DC is observed (Figure 6B and Table 1). Since the number of apoptotic cells in the untreated and blank particle-loaded DC groups is not different, the observed increase in apoptosis of rapaMP-loaded

DC is most probably due to the presence of rapamycin. Importantly, it has been previously shown that the presence of soluble rapamycin in DC cultures leads to increase in apoptosis rates similar to what is depicted in figure 6B for rapaMP [169]. In light of this data, it could be concluded that apoptosis alone is not responsible for the effects of rapaMP-loaded DCs when compared to soluble rapamycin.

It should be noted that the observed increase in inhibitory capacity when using rapaMPs is pronounced (Figure 7) despite the fact that the theoretical quantity of rapamycin delivered is equal to or less than the soluble rapamycin group (amounts calculated based on encapsulation efficiency and cumulative release from rapaMPs). It is likely that this superior inhibitory effect is due to greater intracellular availability of the drug, as it is known that rapamycin inhibits DC function through its action on an intracellular protein kinase [30, 32].

One possible mechanism by which the function of DC is altered upon rapaMP phagocytosis is the limited expression of certain necessary stimulatory modalities on the cell surface (Figure 8). It is well known that activation of T cells by DC requires the expression of MHC (signal 1) in the presence of other co-stimulatory molecules (signal 2). Flow cytometric analysis reveals that the expression of both MHC-II and co-stimulatory molecules (CD86 and CD40) is substantially lower in DC that have taken up rapaMPs (Figure 8). Although it cannot be ruled out that rapaMPs also affect DC in other ways, agents which specifically down-regulate co-stimulation have been identified as promising candidates for clinical transplantation immunotherapy [31, 65] and current phase I clinical trials are underway for autoimmune Type I diabetes that utilize antisense oligonucleotides that knock down CD40 and CD86 [102].

The results may also have implications for DC-specific delivery of many other immuno-modulatory agents that act intracellularly, such as aspirin, corticosteroids, and vitamin D_3

analogues [31]. Thus, it is possible that appropriately-sized degradable microparticulates could be used as a modular platform technology to deliver any number of the newly-identified agents that exhibit immuno-modulatory effects through interactions with DC.

In conclusion, this study demonstrates the successful preparation and characterization of rapaMPs that can be phagocytosed by DC. The results show that the release of rapamycin in pH 7.4 and pH 5 buffers lasts for about 2-3 weeks. Additionally, it was observed that rapaMP-loaded DC have a much lower capacity to induce proliferation in T cells than DC exposed to soluble rapamycin, *in vitro*. This effect may be mediated by down-regulation of surface co-stimulatory molecules. These microparticles have the potential to either be preloaded into DC *ex vivo* or to passively target DC *in vivo* as an antigen presenting cell-specific immunotherapy for transplantation and autoimmune diseases.

3.0 BIO-INSPIRED CONTROLLED RELEASE FORMULATIONS FOR THE RECRUITMENT OF REGULATORY T CELLS (TREG)

3.1 INTRODUCTION

The importance of Treg in determining immune homeostasis in the healthy steady state was discussed in detail in Chapter 1 (Section 1.10). This chapter describes the development and testing of a technology that would increase Treg numbers at a local site *in vivo* in an attempt to induce a homeostatic environment.

The absence or increased presence of Treg in specific tissues has been shown to lead to diverse outcomes [126, 170-171]. For example, Treg deficiency is a causative factor for autoimmune disease [171-172]. Conversely, enriched numbers of Treg at tumor sites results in detrimental suppression of tumor-specific immunity [173]. Although it is not clear how Treg reduction in self tissues occurs, there is some indication as to how these cells are enriched in tumors. Specifically, a wide variety of tumors release the chemokine CCL22 [173-174], which is responsible for tumor-specific migration of Treg and corresponding tumor-specific immune evasion.

From the perspective of therapeutic intervention for organ allograft rejection and autoimmune diseases, the following question arises; would it be possible to attract Treg to the site of interest in a fashion similar to that used by tumors, to suppress local immunity? One

potential method of achieving site-specific localization of Treg is the establishment of a CCL22 gradient through controlled release technology. Herein, we demonstrate that a CCL22-based sustained release vehicle can indeed be used for local recruitment of Treg and suppression of immune reactivity.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Female BALB/c and C57Bl/6 mice (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA); FVB mice were obtained from Taconic (Hudson, NY) and FVB.luc+ mice were from Xenogen (Caliper LifeSciences, Hopkinton, MA)[175]. All animals were maintained under specific pathogen free conditions at the University of Pittsburgh. Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use for Laboratory Animals and under Institutional Animal Care and Use Committee-approved protocols.

3.2.2 Microparticle Preparation

Microparticles (MP) containing rmCCL22 (R&D systems, Minneapolis, MN) were prepared as described [176]. Briefly, 200 µl of a 15mM NaCl solution containing 5 µg of CCL22 and 2 mg of BSA was mixed with 200 mg of Poly (lactic-co-glycolic) acid (RG502H, Boehringer Ingelheim, Petersburgh, VA) dissolved in 4 ml of dichloromethane. This mixture was sonicated

for 10 sec (to form the first emulsion of water-in-oil) before being poured into 60 ml of 2% PVA solution (M.W. ~25,000, 98 mol. % Hydrolyzed, PolySciences, Warrington, PA) being homogenized (Silverson L4RT-A) at 3000 rpm. Following homogenization for 1min (to form the second emulsion), the solution was mixed with 1% PVA and the dichloromethane was allowed to evaporate. After 3 hr, the freshly formed MP were centrifuged and washed (x4) in de-ionized (DI) water. The MP were then re-suspended in 5 ml of DI water, frozen on dry ice and lyophilized (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 100mTorr). Fluorescently-labeled CCL22MP (CCL22MP-680) were prepared by adding 50µg of dextran-alexa fluor® 680 (M.W. 10,000, Invitrogen, Carlsbad, CA) to the 200µl aqueous solution containing CCL22.

3.2.3 Microparticle Characterization

The surface morphology of CCL22MP was characterized using a scanning electron microscope (JEOL JSM-6330F, Peabody, MA) and CCL22MP size distribution measured using volume impedance measurements on a Beckman Coulter Counter (Multisizer-3, Beckman Coulter, Fullerton, CA). The amount of CCL22 encapsulated was determined by degrading 5mg of CCL22MP in 10ml of 0.05N NaOH + 0.5% SDS solution for 2 hr, followed by quantifying the amount of chemokine in the supernatant using a CCL22-specific ELISA kit (R&D systems, Minneapolis, MN). *In vitro* release characteristics were measured by suspending ~7mg of CCL22MP in 1ml phosphate buffered saline (PBS) on an end-to-end rotator at 37°C. At different time intervals, the suspensions were centrifuged, the supernatant collected, and the CCL22MP re-suspended in 1ml PBS. The amount of CCL22 in the supernatant was determined by ELISA.

3.2.4 Alloactivated regulatory T cell (AATreg) cultures:

$CD4^+$ $CD25^+$ regulatory T cells (Treg) were isolated from the spleens and lymph nodes of FVB.luc^+ mice using the following procedure. $CD4^+$ T cells were enriched using the Dynal® mouse $CD4^+$ negative selection kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. $CD25^+$ T cells were isolated from the $CD4^+$ cell pool by labeling the cells with anti-CD25-PE, followed by addition of anti-PE beads (Miltenyi) and separating the bead-labeled cells using a MACS ® column (Miltenyi, Auburn, CA). $CD4^+$ $CD25^+$ (enriched Treg) were then cultured for 10 days with immature allogeneic-DC (propagated from the bone marrow of BALB/c mice [177]) in complete media (containing 10% FBS) and 1000 U recombinant human IL-2. At the end of the culture period, anti-CD11c-labeled beads (Miltenyi) were added to the cells and DC removed using an autoMACS^{TM} separator (Miltenyi). The isolated, alloactivated Treg [178] were then prepared for *in vivo* injections.

3.2.5 *In vivo* Treg migration

CCL22MP-680 (2.5µg suspended in 50µl of PBS) was injected into the triceps surae of FVB mice on day -3 or day -6 (Figure 2a or supplementary Figure 3a schematic). BlankMP-680 injections in the contra lateral limb served as an internal control. AATreg were injected i.v. on day 0 ($2x10^6$ cells in 200 µl of PBS), followed by mature allogeneic DC injection ($1x10^6$ cells in 200 µl of PBS). Non-invasive, live animal bioluminescence imaging was performed at defined time intervals using Xenogen Vivovision (IVIS 200, Caliper LifeSciences, Hopkinton, MA). Luc^+ Treg were detected by injecting 200 µl of luciferin (Caliper LifeSciences) 10 min prior to imaging. The position of each mouse remained unchanged, while both fluorescence and

luminescence imaging was performed. Images acquired from the live animal imaging apparatus were analyzed using Igor Pro Living Image® 2.60.1 (Caliper LifeSciences). Contours of equivalent fluorescence signal from each leg were automatically drawn by the software, and then applied to the luminescence images to determine co-localization of AATreg with particle depots. Statistical analysis was performed using the Student's 't' test on avg. luminescence measurements obtained from the BlankMP-680 and CCL2MP-680 groups.

3.2.6 Allogeneic cell transplant studies

Either CCL22MP-680 or BlankMP-680 was injected subcutaneously into FVB mice 4 days prior to the implantation of allogeneic cells. Transgenic luciferase expressing lewis lung carcinoma cells ($1x10^6$; originally derived from C57Bl\6 mice) were injected at the sites of particle injection (area of particle localization was determined using fluorescence imaging) in 100 µl of PBS. At defined time intervals bioluminescence imaging was performed as described previously. For analysis of acquired images, equivalent circles enveloping the area of luminescence signal were drawn and average luminescence (photons/sec/cm^2/Sr) measured (avg. luminescence measurements negate any differences in signal that may arise due to variations in selection of the area of luminescence). Data are reported as normalized avg. luminescence, where the luminescence intensity measured from any given mouse on any given day was divided by the luminescence intensity measured for the same mouse on day 1. Statistical analysis was performed on the normalized data using the Mann-Whitney U test.

3.3 RESULTS AND DISCUSSION

Sustained release of CCL22 was achieved by loading the chemokine into degradable poly (lactic-co-glycolic) acid-based microparticles (CCL22MP). Scanning electron micrographs of intact MP indicate that they are spherical and slightly porous (Figure 9a).

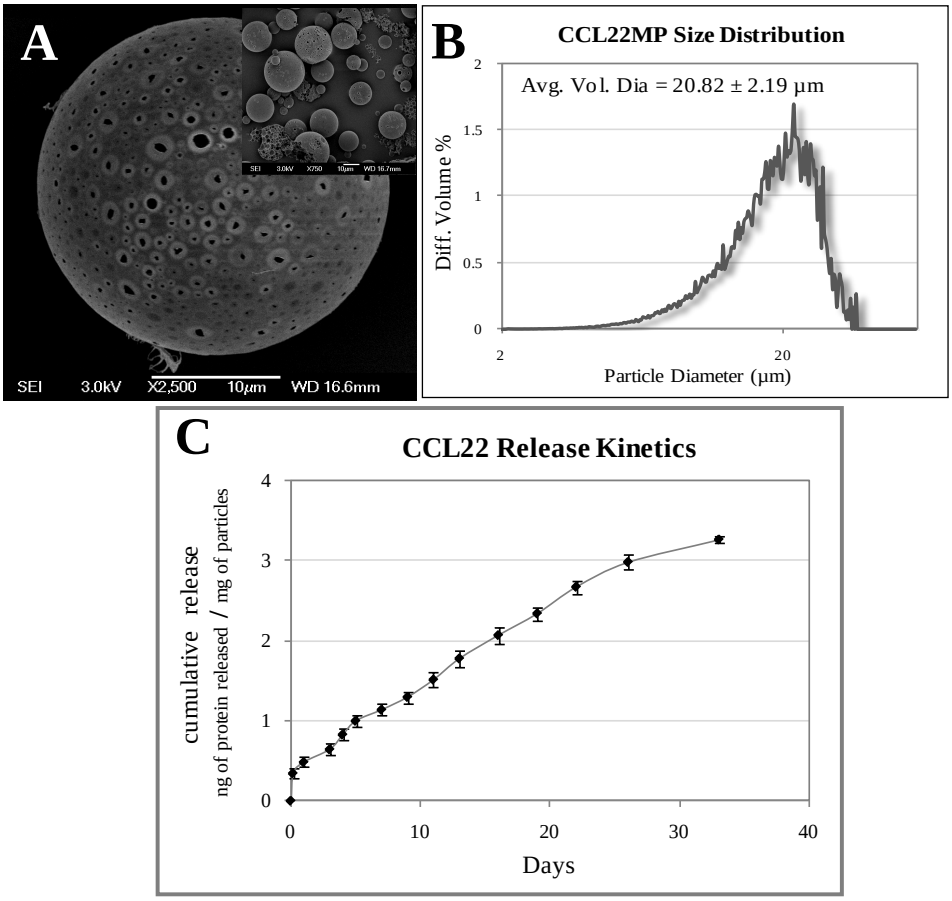

Figure 9: CCL22MP characteristics.

A – scanning electron micrograph of an intact CCL22MP showing its external porous structure. Inset is zoomed-out detail of main image. **B** - representative volume-averaged size distribution of CCL22MP; average volume diameter measurements ± SEM are based on n=6 particle sets. **C** – release kinetics of CCL22MP as measured in PBS; errors bars represent cumulative SEM based on n=3 experiments.

The surface of CCL22MP was specifically formulated to be porous, to allow continuous release (without periods of lag) of chemokine (Figure 9b), as guided by new mechanistic descriptions of how controlled release of proteins occurs in such systems [159-160]. Further, the particles were designed to be large enough to avoid uptake by phagocytic cells and to prohibit their movement across vascular endothelium, with consequent immobilization at the site of injection (Figure 9c).

In order to test the ability of CCL22MP to attract Treg, an *in vivo* adoptive transfer model coupled with non-invasive live animal imaging was used. Fluorescently labeled CCL22MP were injected into the *triceps surae* of normal FVB mice followed by i.v. infusion of *ex vivo*-alloactivated Treg [178] (AATreg) that constitutively express the luciferase gene. The migration pattern of these bioluminescent AATreg was studied following the injection of non-labeled mature allogeneic dendritic cells (DC), which provide an activation stimulus (Figure 10a). Soon after DC stimulation, a significantly greater number of AATreg was recruited to the site of CCL22MP injection (Figure 10b) compared to an internal control of microparticles lacking CCL22 (BlankMP). Further, upon analyzing the kinetics of migration, we determined that residence time of Treg at the site of recruitment was transient (Figure 10c). This type of recruitment pattern is not uncommon and has been observed for other inflammatory cell types [179]. Nevertheless, we did consider the possibility that this transient response may be a result of short-term release of CCL22 from the MP *in vivo* (<10 days). However, delaying adoptive transfer of AATreg post-CCL22MP injection revealed that these cells could also be recruited at later time points (Figure 11), and that the residency remained transient. It is also possible that the absence of antigen-specific stimulation at the site of CCL22MP injection led to the decline in AATreg. Presumably, the presence of Treg could be extended in a stimulatory micro-environment, a hypothesis we are currently testing.

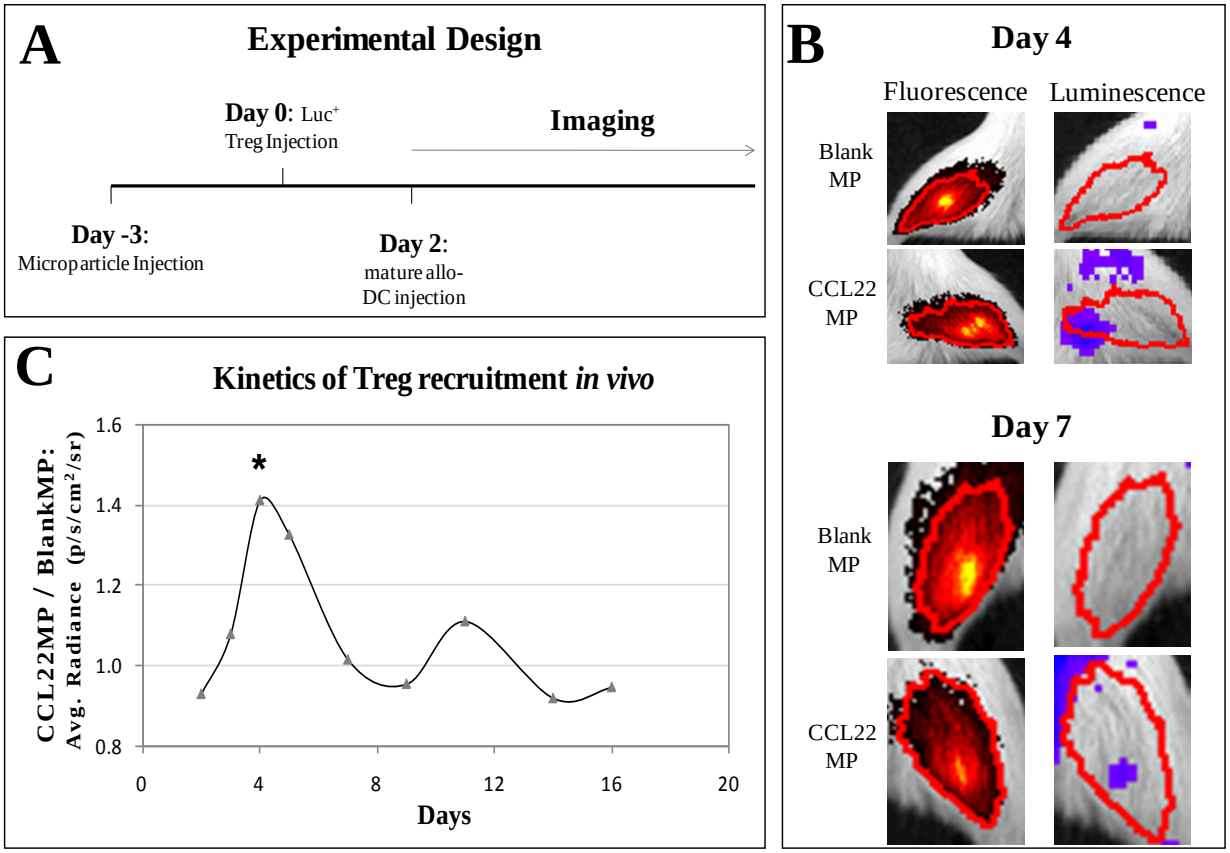

Figure 10: *In vivo* migration of Treg towards CCL22MP.

A – schematic describing the design of the live animal imaging experiment; 'Luc$^+$ Treg' indicates Treg isolated from transgenic FVB.luc$^+$ mice that constitutively express the luciferase gene. **B** – representative fluorescence (red-gold) and luminescence images (blue-yellow) showing localization of particles and Treg, respectively; fluorescence images were used to outline (red-line) areas of particle localization (Igor Pro Living Image® 2.60.1) and these outlines were super-imposed on luminescence images taken with the mouse in identical position to demonstrate co-localization of Treg and CCL22MP. **C** – kinetics of Treg migration towards the injected MP; average radiance measurements obtained from the luminescence images are displayed as a ratio of CCL22MP to BlankMP based on n=6 mice ± SEM at all time points except, day 3 and day 5, where n=3 ± SEM. * indicates p = 0.04 comparing average radiance measurements obtained from CCL22MP with BlankMP using a two-tailed, paired Students't' test.

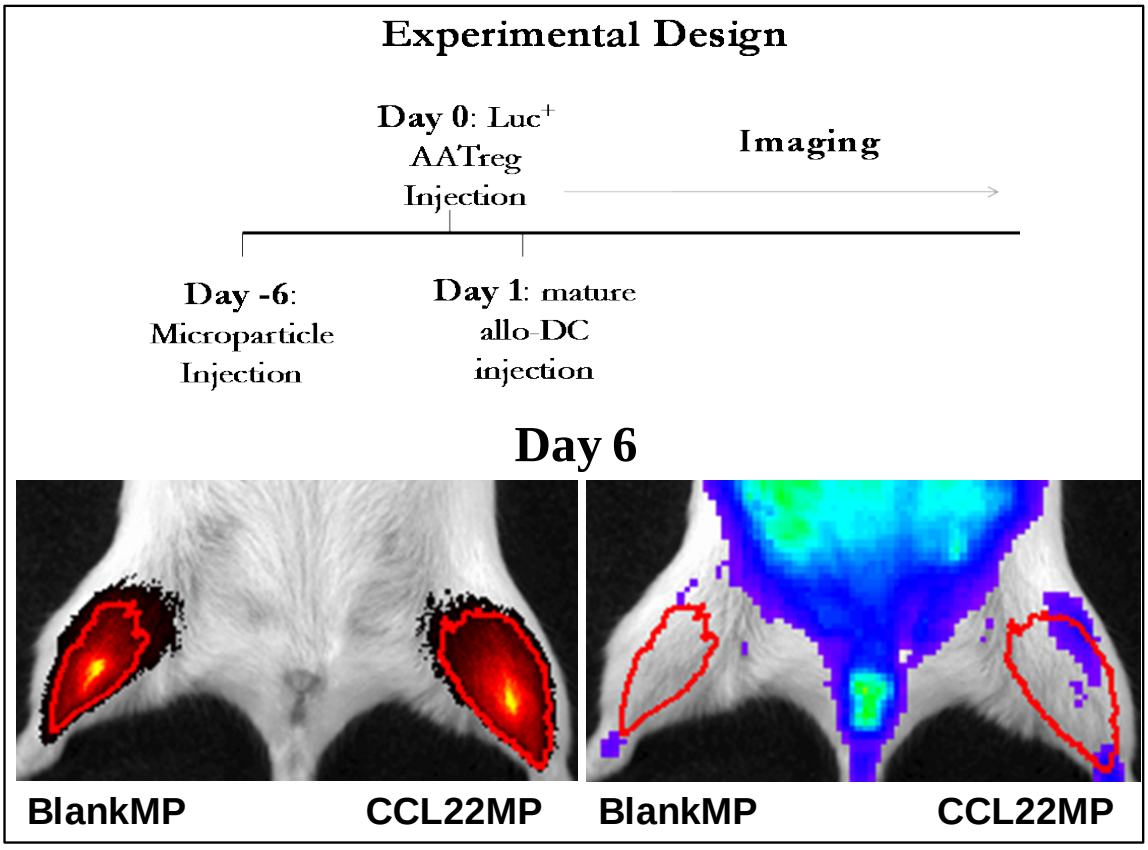

Figure 11: Delayed *in vivo* migration experiments.

Schematic describing the design of the delayed migration experiment - AATreg were adoptively transferred i.v. 6 days after injection of CCL22MP. Representative fluorescence (left) and luminescence (right) images were obtained 6 days after AATreg infusion showing that the cells migrated toward and co-localize with CCL22MP at later time points (12-13 days post particle injection) as well.

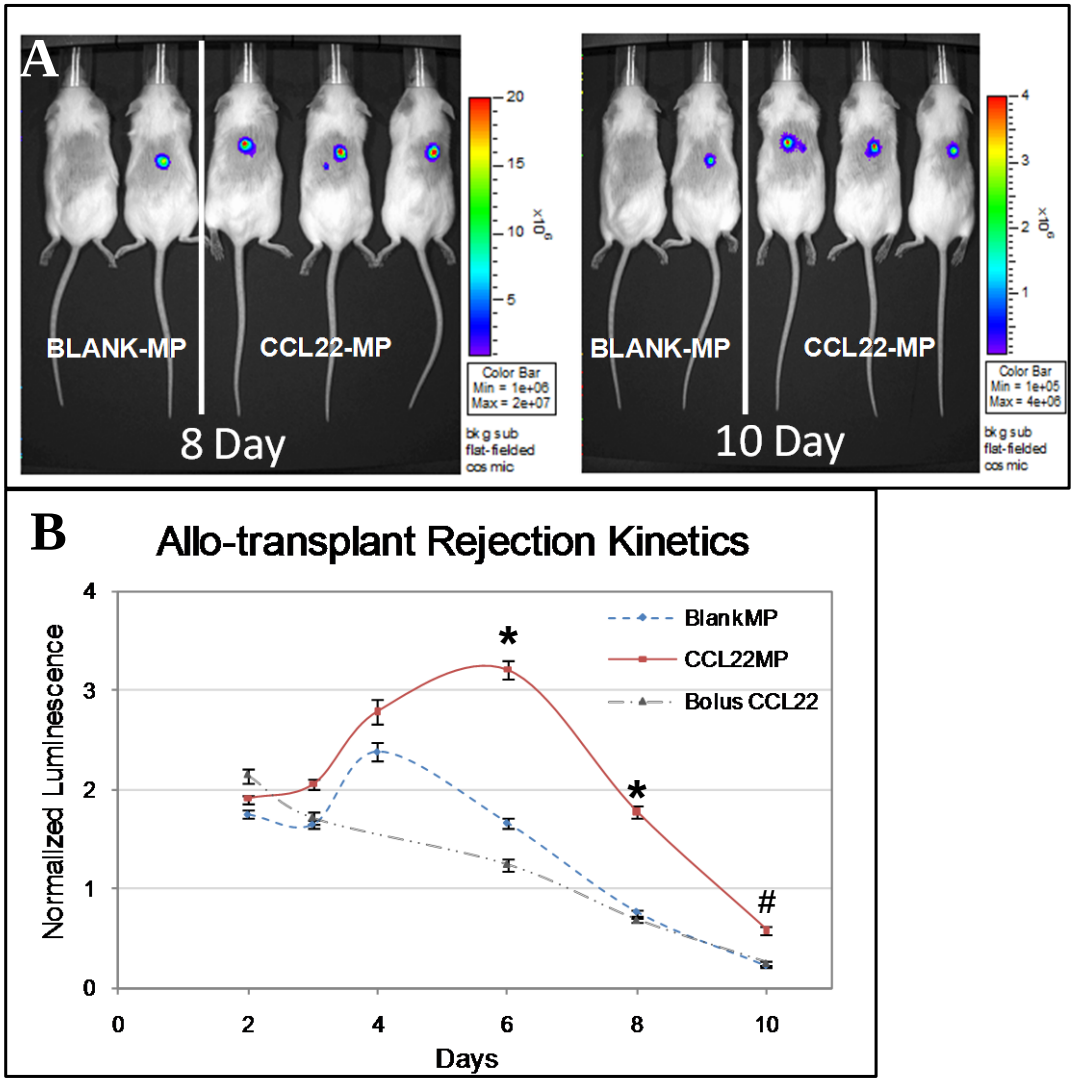

Figure 12: CCL22MP delays rejection of allo-transplants.

A – representative luminescence images of mice injected with particles and luciferase-expressing allogeneic lewis lung carcinoma (LLC) cells. **B** – quantitative analysis of the kinetics of allo-transplant rejection; normalized luminescence was calculated by determining the ratio of luminescence on any given day to the luminescence on day 1 after LLC cell implantation. Error bars indicate SEM based on n = 11 mice (for BlankMP and CCL22MP groups) and n = 5 mice for the bolus CCL22 group. * indicates $p < 0.05$ based on Wilcoxon rank-sum test for null hypothesis that normalized luminescence for CCL22MP is the same as that of BlankMP or bolus CCL22, and # $p < 0.03$ for comparison between CCL22MP and BlankMP only.

Although tracking populations of endogenous Tregs is much more difficult than adoptively transferred luminescent Tregs, it is conceivable that their end effects on the local microenvironment can be readily detected. Indeed, tumors increase the likelihood of their survival through release of CCL22 and corresponding recruitment of endogenous Tregs [173-174]. For this reason, we wished to determine if we could reproduce this effect in an allogeneic cell transplant model.

Specifically, allogeneic luciferase-expressing Lewis lung carcinoma cells (which do not produce CCL22 endogenously) were implanted subcutaneously into mice at the site of CCL22MP injections (or for comparison BlankMP or Bolus CCL22 as controls), and the time to rejection was recorded using non-invasive live imaging. In support of our hypothesis, we observed that the rejection rates were significantly slower in the CCL22MP group when compared to both bolus CCL22 and BlankMP controls (Figure 12a and 12b).

Potential therapeutic implications of a degradable controlled release formulation capable of recruiting Treg *in vivo* are manifold. One obvious application is the use of CCL22MP in combination with an infusion of Treg expanded *ex vivo*. Current pre-clinical data suggest that freshly-isolated or *ex vivo*-expanded Treg infusion can prevent organ transplant rejection or suppress autoimmune diseases. However, challenges such as obtaining adequate numbers and highly-purified populations of Treg have hindered progress into clinical trials [138, 180]. Using formulations that release CCL22, it may be possible to lower the numbers of injected Treg, or potentially use populations with lower purity. Another use of CCL22MP would be to attract endogenous Treg populations, wherein these formulations have the potential to function as “off-the-shelf” therapeutics for the treatment of a wide variety of disorders associated with unrestrained immune reactivity.

A potential drawback of using a CCL22 sustained release vehicle is that the receptor for this chemokine (CCR4) is expressed on both activated Treg and activated effector T cells [181], which suggests that both these cell populations would be attracted towards CCL22MP. Yet *in vivo* studies suggest that CCL22 production associated with tumors [174, 182] or long-surviving allografts [183] result in recruitment of Treg leading to local immunosuppression. One possible explanation for these results is that Treg express significantly more CCR4 than effector T cells [182] (Figure 13).

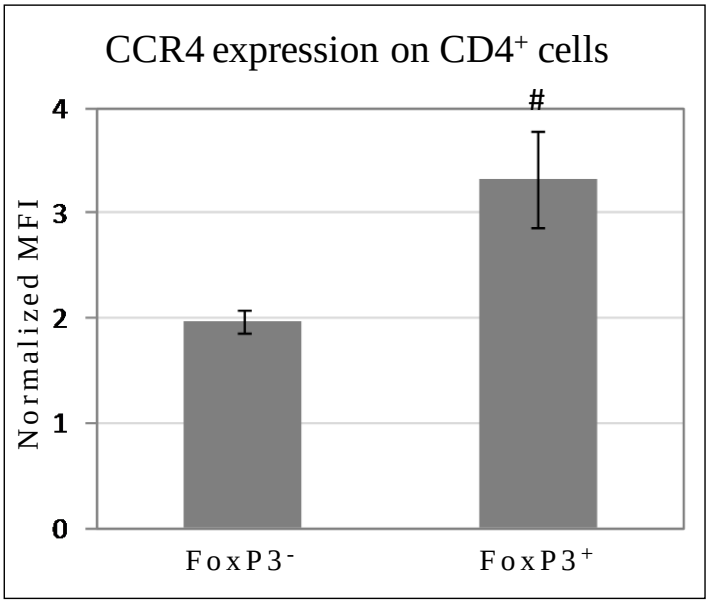

Figure 13: CCR4 expression is higher on Treg.

Surface expression of the chemokine receptor CCR4 was determined on FoxP3 expressing $CD4^+$ T cells (Treg) and FoxP3 $^-$ $CD4^+$ T cells (effector T cells). MFI indicates median fluorescence intensity. Normalized MFI was calculated by determining the ratio of MFI on CCR4 stained cells to the MFI on the isotype stained cells. # indicates $p<0.005$; $n = 5$ (based on 2 independent experiments).

Further, it has been suggested that an optimal ratio of Treg to effector T cells, and not complete absence of effector T cells, is necessary for effective local suppression of immunity [172, 180]. Regardless, if local effector T cells abrogate a suppressive environment, CCL22MP can easily be modified to simultaneously release immunosuppressive agents (such as rapamycin as described in Chapter 2 and [29]) to inhibit these effector T cells *in situ*, thereby assisting Treg to control adverse immune responses.

In conclusion, we demonstrate that site-specific attraction of Treg leading to local immunomodulation can be achieved *in vivo* using CCL22MP. These bio-inspired controlled release formulations are particularly attractive as modular platforms for therapeutic development, as well as tools to study Treg-dependent modulation of immune responses *in situ*.

4.0 CHARACTERIZING THE PHENOTYPE AND FUNCTIONAL CAPABILITIES OF RAPAMYCIN INDUCED REGULATORY T CELLS

4.1 INTRODUCTION:

Treg-based therapies are widely regarded as promising treatment options for autoimmunity and transplant rejection [138, 180, 184]. Currently, several therapies involving the use of *ex vivo* expanded Treg are being tested in clinical trials [138, 185]. However, there are significant barriers to *ex vivo* Treg-therapies such as difficulty in isolating pure populations of these rare cells and expanding them to sufficiently large numbers while maintaining their phenotype and function [138, 140].

One possible alternative to circumvent these issues is to generate adaptive or induced Treg (iTreg) from the patient's own naïve T cells either *ex vivo* or *in vivo*. Past reports have demonstrated that IL-2 and transforming growth factor β1 (TGF- β) can induce Treg phenotype and functional characteristics in naïve T cells upon *in vitro* stimulation [84, 186]. However, TGF-β induced Treg (TGFβ-iTreg) have been shown to be unstable in long term *in vitro* cultures and upon antigenic re-stimulation [128]. Additionally, the presence of inflammatory cytokines such as IL-6 can antagonize TGF-β mediated induction of Treg [187-188], making the presence of such inflammatory mediators a potential impediment to inducing Treg *in vivo* at the site of the disease.

Numerous reports in the literature suggest that these problems can be overcome through the use of small molecules that work in concert with TGF- β to induce Treg. For example, all-trans retinoic acid (RA) is known to potently synergize with IL-2 and TGF-β to induce FoxP3 expression in naïve T cells [189-191] and allow for induction of Treg even in the presence of inflammatory cytokines. Thorough characterization of the phenotype and function of RA induced Treg (RA-iTreg) demonstrates that they are better suppressors and more stable than TGFβ-iTreg [190, 192]. Nevertheless, their use is limited by the fact that RA-iTreg are known to primarily migrate to the mucosal tissues in the gut [189-190]. Further, recent evidence suggests that depending on the immunological microenvironment RA can induce inflammation instead of tolerance [193]. Also, RA has been shown to induce hypervitaminosis-A upon local administration [194-195], and hence it would be difficult to use this combination (cytokines + RA) to induce Treg *in vivo*. Another small molecule that synergizes with IL-2 and TGF-β to induce FoxP3 expression in naïve T cells is rapamycin (rapa) [196-198]. Although it has been demonstrated that like RA, rapa can induce Treg even in the presence of IL-6 [196], the *in vitro* phenotype and functional capabilities of rapa induced Treg (rapa-iTreg) are yet to be characterized.

In this study we compare and contrast the phenotype (expression of canonical Treg markers and migratory surface markers), functional capabilities and stability upon *in vitro* re-stimulation of TGFβ-iTreg, RA-iTreg and rapa-iTreg. Our data suggests that the combination of IL-2, TGF-β and rapa is capable of inducing functional and stable Treg populations with largely a lymphoid tissue homing capacity. In addition, we describe the *in vitro* characteristics of Treg generated by combining IL-2, TGF-β, RA and rapa (RA+rapa-iTreg).

4.2 METHODS

4.2.1 Animals

Six-eight week old C57Bl/6 and B6.SJL-Ptprca/BoyAiTac (CD45.1) were purchased from Taconic and used within one month. B6(Cg)-Tyrc-2J/J (albino C57Bl/6 mice) were purchased from Jackson Laboratories. C57Bl/6.Luc+ mice were a kind gift from Dr. Stephen Thorne (Dept. of Surgery, University of Pittsburgh). All animals were maintained under specific pathogen free conditions. Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use for Laboratory Animals and under Institutional Animal Care and Use Committee-approved protocols.

4.2.2 Materials

Mouse CD4 negative isolation kit, αCD3/αCD28 labeled beads (aAPC Dynal ®) and Vybrant CFDA-SE cell tracer kit were from Invitrogen Corporation (Carlsbad, CA, USA). Recombinant mouse IL-2 (R&D systems, Minneapolis, MN, USA), recombinant human TGF-β1 (CHO cell derived, PeproTech, Rocky Hills, NJ, USA), all-trans-retinoic acid (Sigma, St. Louis, MO, USA), and rapamycin was from LC labs (Woburn, MA, USA). The following antibodies were purchased from eBioscience (San Diego, CA, USA): CD4 (L3T4), FoxP3 (FJK-16s), CD45.1 (A20), CD103 (2E7), CD25 (PC61.5), GITR (DTA-1), FR4 (eBio12A5), CCR7 (4B12) and CCR9 (eBioCW-1.2). CTLA4 (UC10-4B9) was from BioLegend (San Diego, CA, USA). Anti-PE microbeads were obtained from Miltenyi Biotec (Auburn, CA, USA).

4.2.3 T cell isolation

Spleen and lymph nodes were dissected from mice, and single cell suspensions were prepared using mechanical digestion. Following RBC lysis, $CD4^+$ cell isolation was performed using the CD4 negative isolation kit (Invitrogen) as per manufacturer's instructions. To enrich for $CD25^-$ cells, $CD4^+$ cells were incubated with anti-mouse CD25-PE antibody (eBioscience) followed by addition of anti-PE microbeads (Miltenyi). Bead-bound $CD25^+$ cells were isolated by passing cells through a magnetic column. Unbound $CD4^+$ $CD25^-$ cells were separated and used further to induce regulatory T cells (Treg).

4.2.4 Treg Induction

Freshly isolated naïve $CD4^+$ $CD25^-$ cells were cultured with aAPC Dynal ® beads at a 2:1 (dynal:cell) ratio in the presence of 10 ng/ml IL-2, 20 ng/ml TGF-β1 and/or 3 ng/ml (10nM) all-trans-retinoic acid (RA) and/or 10 ng/ml rapamycin (rapa). To obtain effector T cells (Teff), $CD4^+$ $CD25^-$ cells were cultured with aAPC Dynal ® beads and 10 ng/ml IL-2 only. Cell cultures were carried on for 4 days, and cells were subsequently separated from the magnetic Dynal® beads. To determine induction of Treg phenotype, FoxP3 staining and flow cytometry (BD-LSRII) was performed at the end of the 4-day culture period as per manufacturer's instructions (eBioscience).

4.2.5 *In vitro* suppression assay

Freshly isolated naïve $CD4^+$ $CD45.1^+$ cells were stained with CFSE (invitrogen, as per manufacturer's instructions) and co-cultured with induced Treg (generated as described above) at different ratios in 96-well plates. The number of naïve $CD4^+$ $CD45.1^+$ cells was always kept constant at 50,000 cells / well. For stimulation, 25,000 aAPC Dynal ® beads per well were used (2:1, naïve cell:dynal ratio). Co-cultures were carried out for 4 days, followed by staining for flow cytomtery.

4.2.6 *In vivo* suppression assay:

A modified version of the colitis model [199] was used for the *in vivo* suppression assays. Briefly, 100,000 freshly isolated naïve $CD4^+$ $CD25^-$ cells were co-transferred with 50,000 iTreg (generated under different conditions from $CD45.1^+$ mice) (naïve:iTreg) in 200 µl PBS per animal (in immune-deficient $RAG^{-/-}$ mice) through the tail vein. The mice were weighed regularly and monitored for any adverse signs of disease. At 22-days post cell injection, mice were euthanized and the spleen, mesenteric lymph nodes and colon collected for analysis.

4.2.7 Testing iTreg stability *in vitro*

iTreg generated under different conditions and effector T cells (T_{eff}, generated by stimulating naïve T cells in the presence of IL-2 only) were obtained from 4 day cultures and rested in 10 ng/ml IL-2 for 2 days. Following resting, cells were cultured along with aAPC Dynal ® beads as stimulators: (i) for Dynal ® only (no T_{eff} and no factors) group, 100,000 iTreg were cultured with

200,000 Dynal along with 10 ng/ml IL-2. (ii) for Dynal ® + T_{eff} (no factors) group, 50,000 iTreg were cultured with 50,000 T_{eff} and 100,000 Dynal along with 10 ng/ml IL-2. (iii) for Dynal ® + factors (no T_{eff}) group, 100,000 iTreg were cultured with 200,000 Dynal and respective factors at concentrations described above. (iv) for Dynal ® + T_{eff} + factors group, 50,000 iTreg were cultured with 50,000 T_{eff} and 100,000 Dynal and respective factors. Re-stimulation experiments were carried out for 4 days and cells were subsequently stained and analyzed by flow cytometry.

4.2.8 *In vivo* migration experiments

iTreg generated from naïve $CD45.1^+$ $CD4^+$ $CD25^-$ cells were injected in CD45.2 mice at a concentration of 2 million cells in 200 µl PBS per animal (tail vein). Three days following injection, mice were euthanized and the spleen, cervical lymph nodes and mesenteric lymph nodes collected. Single cell suspensions from each of these tissues were prepared, stained for different markers and analyzed by flow cytometry. For the *in vivo* live animal imaging experiments, iTreg were generated from C57Bl/6.Luc+ mice and injected in albino C57Bl/6 mice at a concentration of 1 million cells in 200 µl PBS per animal (tail vein). At defined time-points, mice were injected with 200 µl luciferin (30 mg/ml) and imaged using the IVIS 200 (Xenogen VivoVision, Caliper Life Sciences, Hopkinton, MA, USA). Luminescent images were analyzed and quantified using Igor Pro Living Image® 2.60.1 (Caliper Life Sciences).

4.2.9 Statistical Analysis

Mean ± standard deviation values are reported in all figures unless otherwise indicated. Error bars in graphs represent standard deviations. A paired or unpaired Student's 't' test was used for statistical comparison between any 2 given samples unless otherwise indicated.

4.3 RESULTS:

4.3.1 Small molecules enhance ability of TGF-β to induce and maintain functional Treg

Consistent with prior studies, we observed that the presence of either RA or rapa enhances the ability of IL-2 and TGF-β to induce FoxP3 expression in naïve T cells (Figure 14A, 14B and 15) [189-190, 197-198]. The same effect was observed when cells are cultured in the presence of a combination of IL-2, TGF-β, RA and rapa. Cells cultured under all these different conditions also expressed canonical Treg markers such as, FR4, CTLA4, GITR and CD25 (Figure 14C), suggesting that these cells are induced Treg (iTreg). Interestingly, the expression level of one of these markers, FR4 (which along with CD25 has been identified as markers that can help distinguish activated effector T cells from Treg [121]), was significantly greater in rapa-iTreg and RA+rapa-iTreg when compared to RA-iTreg (Figure 16).

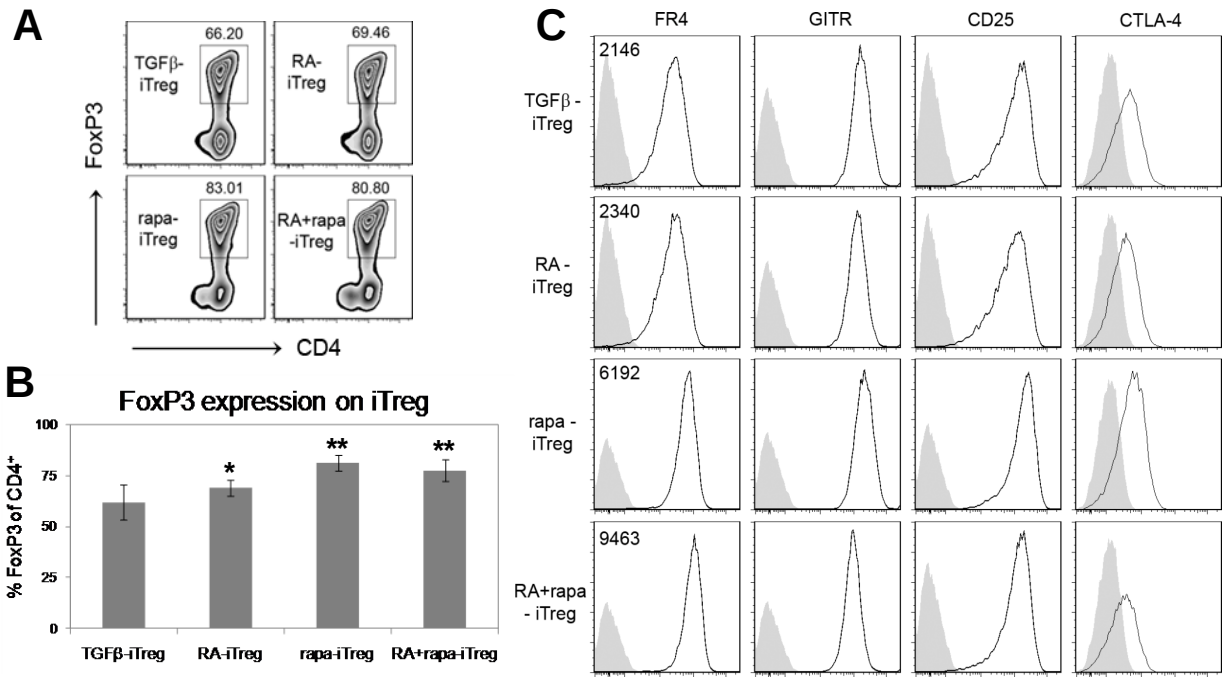

Figure 14: RA and rapa enhance TGFβ's capability to induce Treg phenotype.

A - flow cytometry density plots indicating % of $CD4^+$ cells that express FoxP3 (representative of 6 independent experiments). Plots were generated after gating on $CD4^+$ cells. **B** – Quantitative analysis of the % of $CD4^+$ that express FoxP3. * indicates $p<0.05$, and ** indicates $p<0.01$ when specified group was compared to the TGFβ-iTreg group using the paired Student's 't' test (based on $n\geq6$). **C** – Representative histograms (at least 2 independent experiments) for canonical markers expressed on Treg. Plots were generated after gating on $CD4^+$ $FoxP3^+$ cells. Numbers on plots represent median fluorescent intensities. Filled gray histograms represent isotypes.

TGFβ (ng/ml) →

rapa (ng/ml) ↓	0	0.1	1	5	20
0	0.52	0.61	2.82	18.16	53.26
1	1.91	3.04	8.89	34.41	76.22
10	3.45	5.31	14.48	51.08	78.27
100	4.13	7.8	22.05	54.19	80.36

TGFβ (ng/ml) →

RA (nM) ↓	0	0.5	5	20	80
0	1.69	8.08	28.52	27.42	34.65
0.1	2.71	11.98	35.18	28.33	37.89
1	2.64	10.5	44.71	42.25	44.66
10	2.27	9.36	42.22	45.97	44.27
100	2.23	10.01	50.71	43.43	42.54

Figure 15: Ability of RA and rapa to induce Treg in combination with TGF-β is dose dependant.

Tabulated numbers represent % of CD4+ that express FoxP3. Boxes highlighted in red (represent concentrations at which the best FoxP3 induction and Treg proliferation was observed) indicate the concentrations of TGF-β, RA and rapa used in all experiments described in the study.

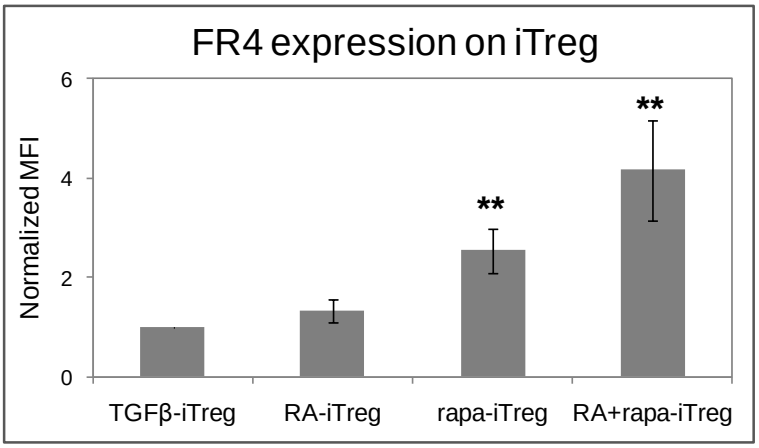

Figure 16: Rapa enhances induction of FR4 expression.

Quantitative analysis of FR4 expression on iTreg generated under different conditions. Median fluorescence intensity (MFI) was determined after gating on the $CD4^+$ $FoxP3^+$ population. MFI values were normalized to TGFβ-iTreg FR4 expression. ** indicates $p<0.01$ when specified group was compared to the RA-iTreg group.

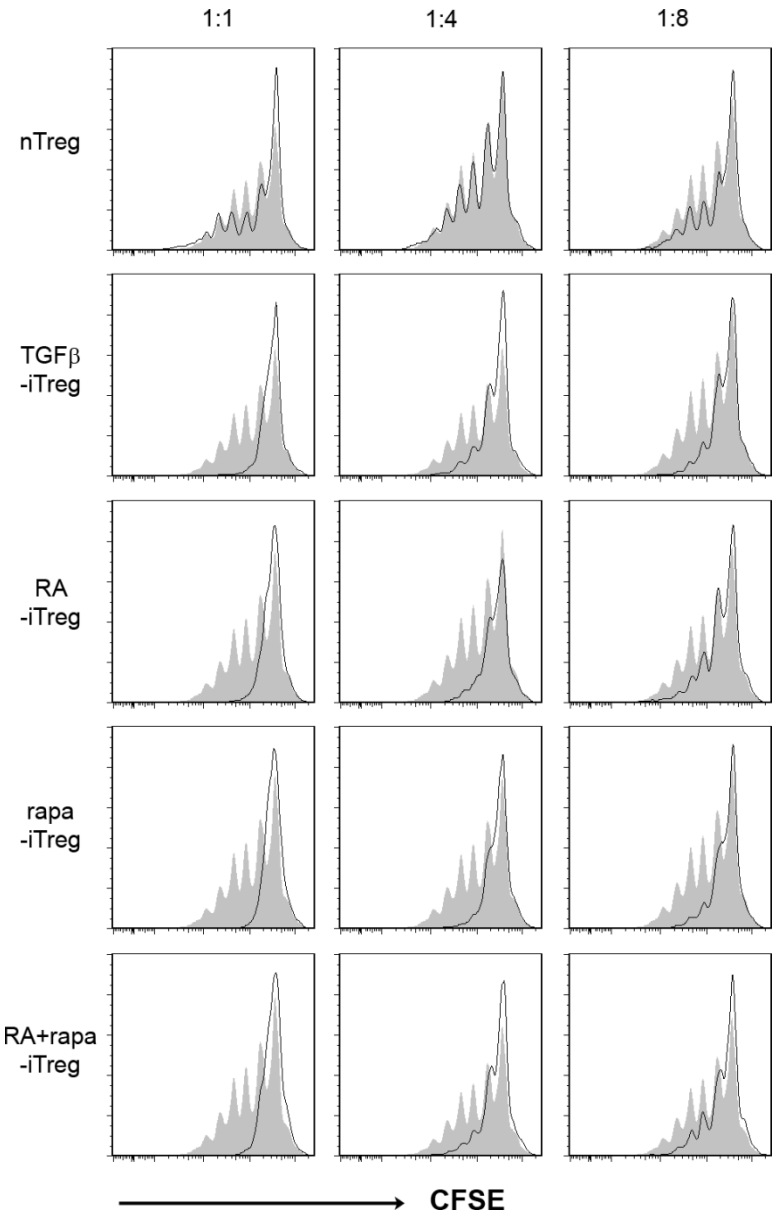

Figure 17: iTreg suppress in vitro naïve T cell proliferation.

Treg capability of suppression determined using the CFSE dilution assay. Filled histograms (gray) represent the proliferation capacity of naïve T cells only (in the presence of stimulation). Black lines represent the proliferation of naïve T cells in the presence of iTreg under specified conditions. Ratios on the top indicate the ratio of Treg to naïve T cells. Plots are representative of at least 2 independent experiments.

In order to determine the functional capacity of the iTreg, their ability to suppress *in vitro* naïve T cell proliferation and *in vivo* function was tested. Upon co-culture with naïve T cells under *in vitro* stimulation (αCD3/αCD28 labeled Dynal ® beads), iTreg generated under all the different conditions were effective at suppressing naïve T cell proliferation (Figure 17).

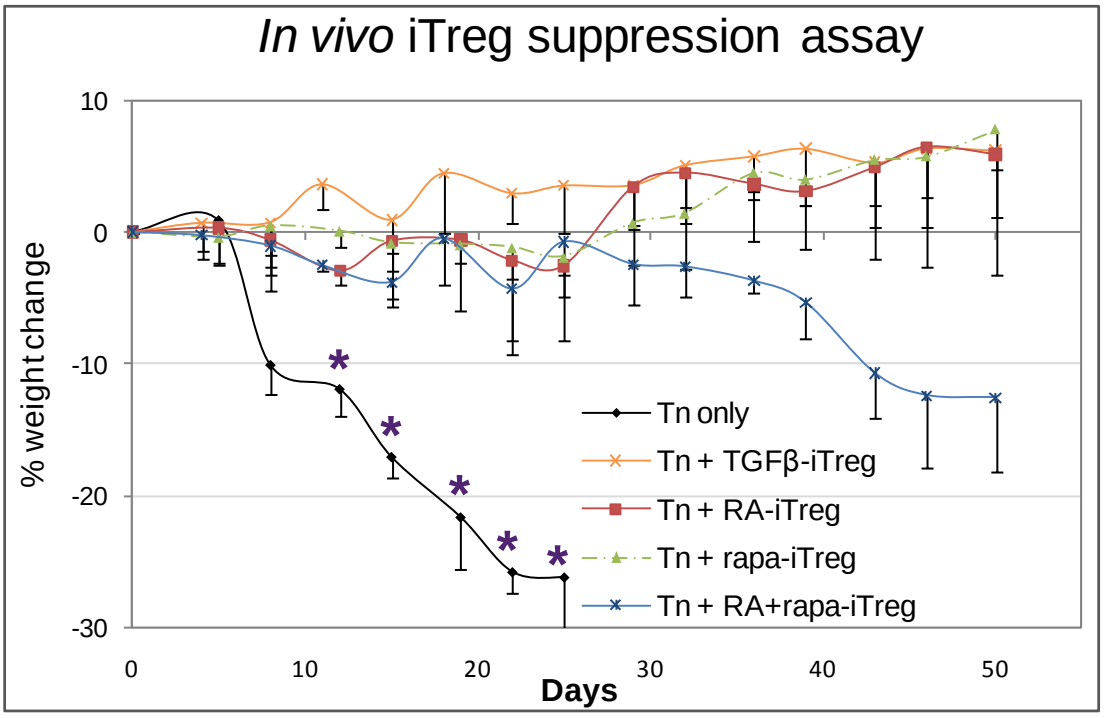

Figure 18: iTreg suppress in vivo naïve T cell activity.

iTreg ability to suppress naïve T cells (Tn) *in vivo* determined as a measure of the development of colitis. Injection of naïve T cells only induced dramatic weight loss in mice while the presence of iTreg prevented this weight loss. Cells were injected at a ratio of 2:1 (naïve T cells to iTreg). * indicates $p < 0.005$ when Tn only group is compared to any of the iTreg injected groups ($n \geq 3$ mice / group till day 25). $n \geq 2$ mice / group after day 25

To examine *in vivo* suppressive capability, the potential of iTreg to prevent T cell mediated induction of colitis in immune-deficient mice [199] was tested. Mice that received only

$CD4^+$ $CD25^-$ (naïve) T cells developed severe colitis with rapid weight loss, while mice that received naïve T cells along with iTreg generated under any of the aforementioned conditions (at 2:1 ratio) were protected from the disease and loss of weight (Figure 18).

4.3.2 iTreg stability upon re-stimulation

It has been demonstrated that following long-term *in vitro* culture, there is a considerable reduction in the percentage of TGFβ-iTreg cells that express FoxP3 [128]. To determine if the same would be true with iTreg generated in the presence of RA and/or rapa, these induced populations were re-stimulated through TCR activation (Dynal®) with IL-2 (no addition of TGF-β, RA or rapa) in either the absence or presence of effector T cells (T_{eff}). Re-stimulation in the absence of T_{eff} led to slightly lowered percentages of FoxP3 expressing cells among the iTreg, while the presence of T_{eff} led to a marked decrease in FoxP3 expressing cells (Figure 19A).

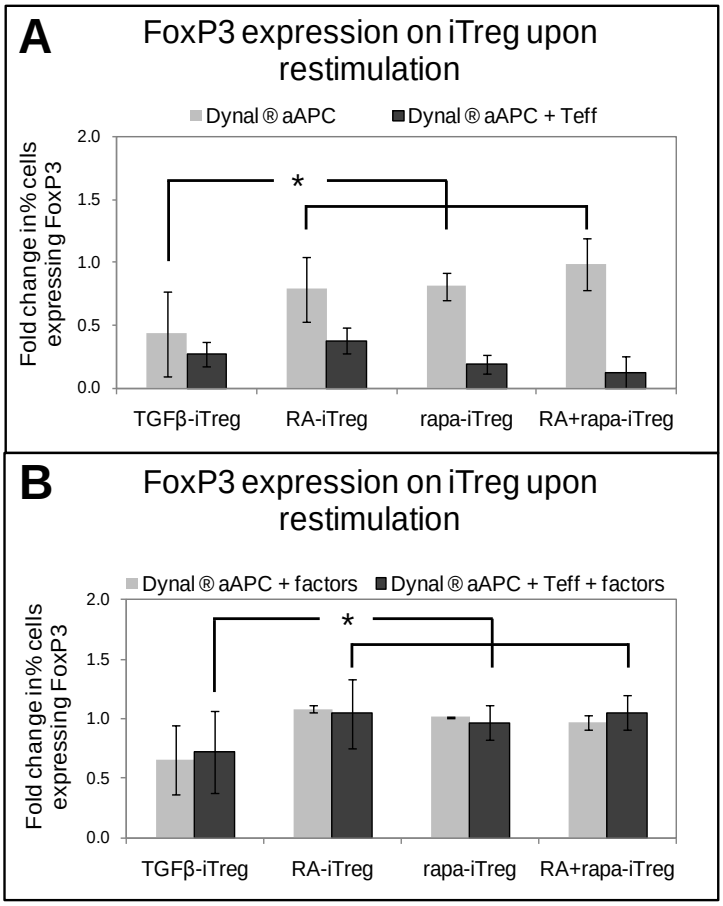

Figure 19: iTreg generated in the presence of RA and/or rapa are more stable than TGFβ-iTreg in long term in vitro cultures.

Quantitative analysis of the fold change in % of iTreg that express FoxP3 after *in vitro* re-stimulation either in the absence of factors **(A)**; and the presence of factors **(B)**. Factors indicate the cytokines and/or small molecules used to generate the iTreg. Data are representative of 4 independent experiments. Fold change is expressed as a ratio between the % of FoxP3 expressing cells at the end of the re-stimulation cultures to the % of FoxP3 expressing cells at the beginning of the cultures.

Interestingly, the presence of the immunosuppressive factors under which the iTreg were generated (a. IL-2, TGF-β, and RA for RA-iTreg; b. IL-2, TGF-β, and rapa for rapa-iTreg; c. IL-2, TGF-β, RA and rapa for RA+rapa-iTreg), prevented the decrease in percentages of FoxP3

expressing cells (Figure 19B). A dramatic decrease in the percentage of cells expressing FoxP3 was observed in TGFβ-iTreg cultured under any of the aforementioned stimulatory conditions.

4.3.3 Expression of migratory receptors on iTreg

RA-iTreg are known to express surface markers specifically associated with migration to the mucosal tissues such as CCR9 and CD103 [189-190]. We observed that rapa-iTreg do not express either of these markers, but express significantly higher levels of CCR7 (Figure 20A and 20B) when compared to RA-iTreg. Interestingly, RA+rapa-iTreg, which also express significantly higher levels of CCR7, appear to contain two distinct iTreg populations; a $CCR9^{+}$ $CD103^{+}$ and a $CCR9^{-}$ $CD103^{-}$ population (Figure 21).

In order to determine if the expression of these receptors would determine *in vivo* homing, iTreg generated from CD45.1 mice were adoptively transferred to healthy CD45.2 mice maintained under homeostatic conditions. As expected, 3 days following adoptive transfers, a significantly greater number of rapa-iTreg and RA+rapa-iTreg were observed in the cervical and mesenteric lymph nodes when compared to TGFβ-iTreg and RA-iTreg (Figure 22).

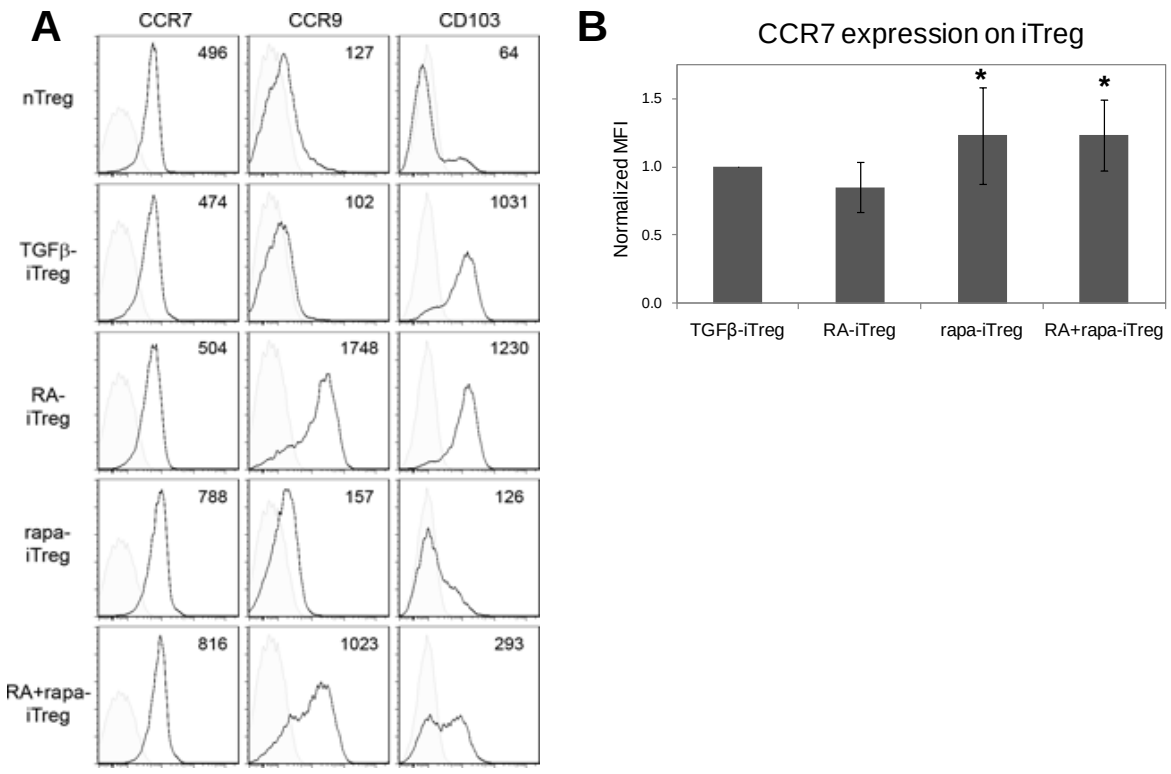

Figure 20: Differential expression of migratory receptors on iTreg generated under different conditions.

A – Histograms depicting expression patterns of different migratory receptors; plotted after gating on $CD4^+$ $FoxP3^+$ cells. Numbers on plot represent MFI values. B – Quantitative analysis of CCR7 expression on iTreg generated under different conditions (MFI values were normalized to TGFβ-iTreg cells). * indicates $p<0.05$ (paired Student's 't' test) when comparing specified group to RA-iTreg group; n=5.

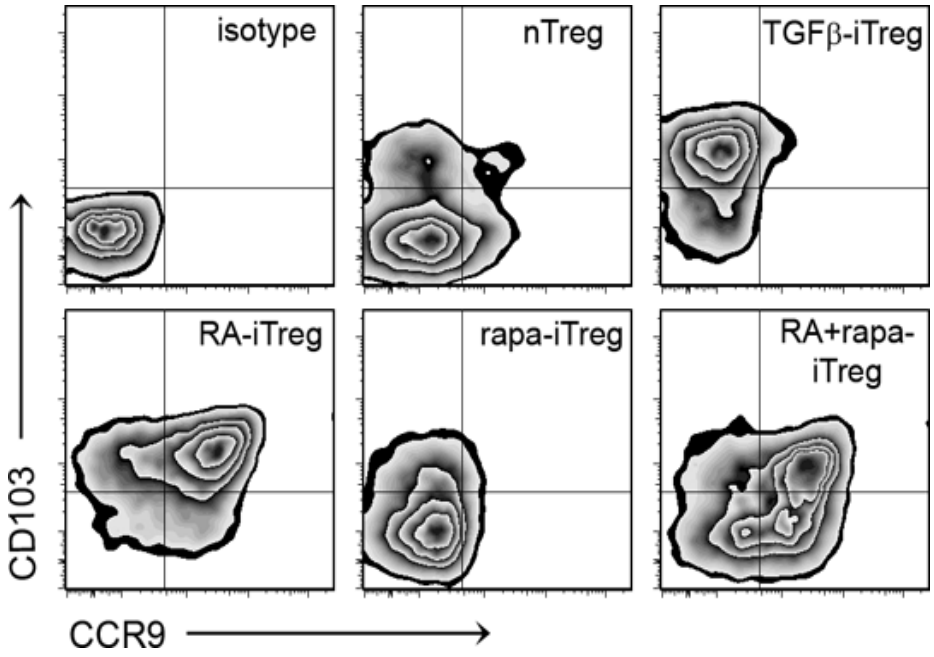

Figure 21: Analysis of surface proteins responsible for migration of T cells to peripheral tissues.

Density plots (flow cytometry) show that presence of RA induces expression of CCR9 on iTreg, while rapa reduces expression of CD103 and CCR9. The RA+rapa-iTreg appear to have two distinct population of cells; one that is $CCR9^{+}CD103^{+}$ and another that is $CCR9^{-}CD103^{-}$.

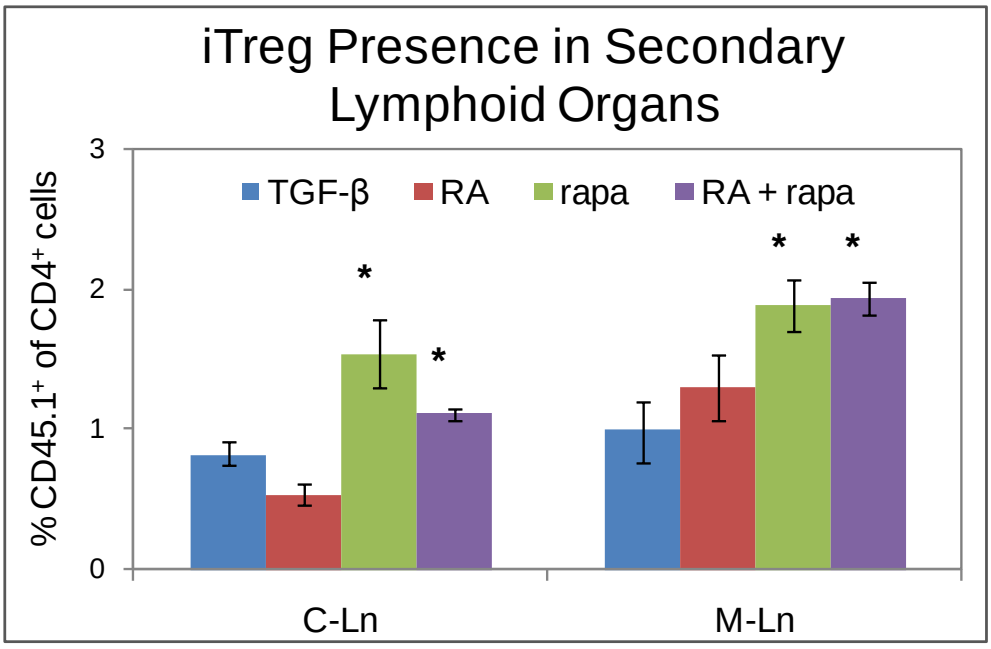

Figure 22: Differential migration pattern of iTreg generated under different conditions. iTreg generated from CD45.1$^+$ were injected into CD45.2$^+$ mice, and after 3 days Spl (spleen), C-Ln (cervical lymph nodes) and M-Ln (mesenteric lymph nodes) harvested to analyze the percentage of CD45.1$^+$ cells. * indicates $p<0.05$ when comparing specified group to either the TGFβ-iTreg or RA-iTreg groups.

Furthermore, we observed that all adoptively transferred iTreg populations maintain their FoxP3 expression capacity (Figure 23). The increased presence of rapa-iTreg in secondary lymphoid organs, and presence of RA-iTreg primarily in the gut, was confirmed using live animal imaging that permits the tracking of cell movement over an extended time-period (Figure 24A and 24B). Additionally, this data suggest that RA+rapa-iTreg migrate both to the secondary lymphoid organs and the gut tissue, although there are considerably greater numbers of cells in the gut tissue (which might be due to the inclusion of mesenteric lymph nodes among the gut tissue in these live animal images).

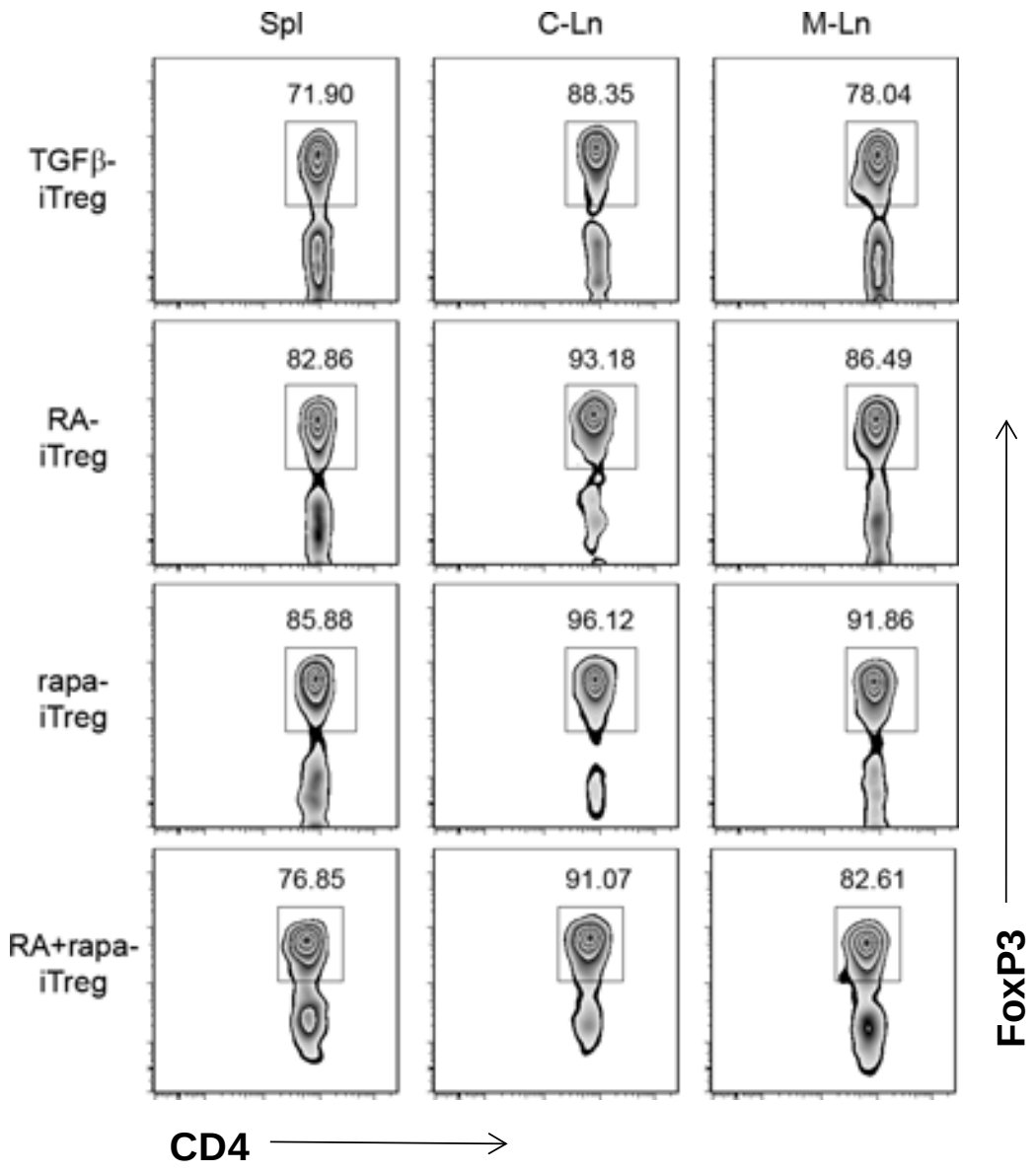

Figure 23: iTreg maintain FoxP3 expression following injection in mice maintained under homeostatic conditions.

Analysis of FoxP3 expression on CD45.1^{+} cells that were injected in wild type CD45.2^{+} mice. Spl indicates spleen, C-Ln indicated cervical lymph nodes and M-Ln indicates mesenteric lymph nodes.

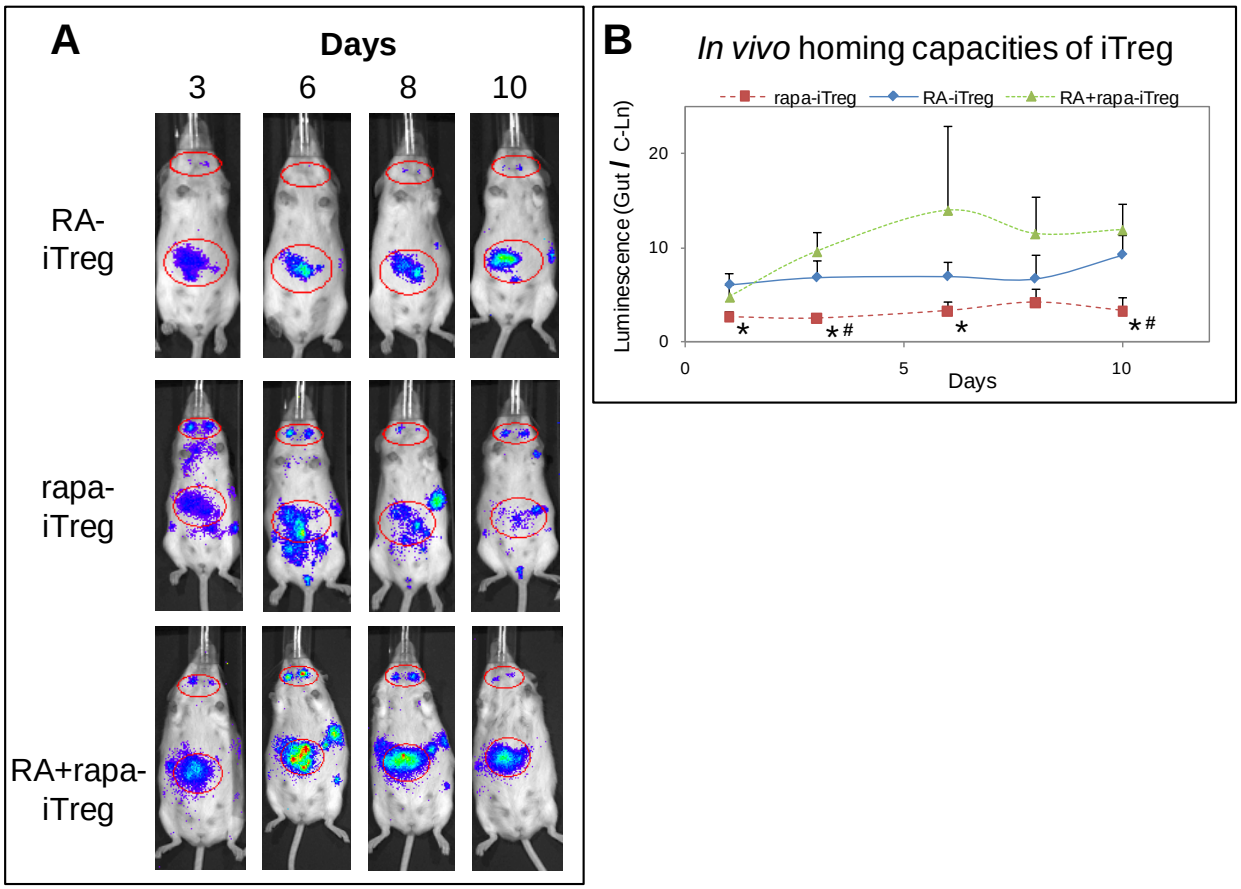

Figure 24: Live animal imaging shows areas of iTreg localization upon injection in vivo.

A – Representative images acquired using the IVIS 200, showing iTreg localization over 10 days. Red ellipses indicate the cervical lymph node and gut area. **B** – A ratio of the average luminescence measurements obtained from the red ellipses depicted as a function of time. * indicates $p \leq 0.05$ when comparing rapa-iTreg with RA-iTreg and # indicates $p \leq 0.05$ when comparing rapa-iTreg with RA+rapa-iTreg. $n \geq 3$ mice for all groups. C-Ln indicates the cervical lymph node area.

4.4 DISCUSSION

Treg can be induced under a variety of different *in vitro* [189-190, 196-197, 200] and *in vivo* [88, 192, 201-202] conditions. Treg induced under different conditions have been reported to have unique characteristics that distinguish them from each other and from naturally occurring Treg. Identification of these unique characteristics provides us insight into their potential to be used in treating inflammatory disorders (such as autoimmunity and allergies) and transplant rejection, or in preventing tumor growth and metastasis [129]. For example, it has been demonstrated that RA can help to convert naïve T cells to Treg. These RA-iTreg are stable under inflammatory conditions and have the potential to prevent inflammation in the gut primarily due to their ability to specifically migrate to these tissues. However, gut homing specificity could potentially be a hindrance to using these cells to treat autoimmunity or transplant rejection at other peripheral sites.

In this study *in vitro* generated rapa-iTreg were directly compared TGFβ-iTreg, RA-iTreg and RA+rapa-iTreg. In many aspects, such as expression of canonical Treg markers (Figure 14), *in vitro* and *in vivo* suppressive capabilities these different iTreg appear to be similar (Figure 17 and 18). However, some noticeable differences between these iTreg were identified. For example, the expression of one of the canonical surface markers, FR4, was significantly greater on the surface of rapa-iTreg and RA+rapa-iTreg when compared to the RA-iTreg (Figure 14 and 16). It has been suggested that expression of this folate receptor may allow for greater survivability and long-term stability of Treg populations in the periphery [121], and it remains to be determined if this is the case with rapa-iTreg and RA+rapa-iTreg.

Most importantly, key differences in the expression of certain chemokine receptors and integrins on the surface of RA-iTreg, rapa-iTreg and RA+rapa-iTreg were observed. As

demonstrated previously, CCR9 and CD103 (surface proteins that direct migration of cells towards the small intestine lamina propria and the epithelium respectively) expression was upregulated on RA-iTreg (Figure 20A). Rapa-iTreg do not express either of these surface proteins, but express the lymphoid organ homing receptor CCR7 at significantly greater levels (Figure 20B). This pattern of expression correlates well with their *in vivo* lymphoid organ homing capacity of rapa-iTreg, while the RA-iTreg primarily migrate to the gut tissue (Figure 22 and 24). RA+rapa-iTreg also express higher levels of CCR7 (Figure 20B), but present as a bimodal distribution of cells, where some cells are $CCR9^{+}$ $CD103^{+}$ and others are $CCR9^{-}$ $CD103^{-}$ (Figure 23). This bimodal population could suggest that cells affected by RA are not influenced by rapa and *vice versa*. It remains to be seen if such an expression pattern allows for the RA+rapa-iTreg to be more efficient at suppressing immune responses (due to their ability to simultaneously migrate to both mucosal tissues and lymphoid organs) *in vivo*, and if such an effect cannot be observed by using a mixture of RA-iTreg and rapa-iTreg.

Additionally, it was observed that although RA and/or rapa induced Treg are more stable when compared conventional TGFβ-iTreg upon re-stimulation, they tend to lose their FoxP3 expression in the presence of stimulation and effector T cells (an *in vitro* mimic of inflammatory conditions). This loss of FoxP3 expression is limited to the presence of effector T cells (Teff) as in the presence of mature dendritic cells such an effect was not observed (data not shown). Nevertheless, the inflammatory effect of T_{eff} is negated in the presence of an immunosuppressive milieu (such as the combination of IL-2, TGF-β, and RA or rapa) and the iTreg maintain their FoxP3 expression even after incubation with T_{eff} (Figure 19). This would suggest that the use of Treg induced in the presence of small molecules might be limited to situations where an immunosuppressive milieu can be established using (for example) low dose immunosuppressive

regimens, or controlled release formulations for immunomodulatory agents. Furthermore, recently it has been suggested that the methylation patterns on the Foxp3 gene locus is a good determinant of long-term Treg stability [128]. It remains to be determined if these iTreg differ in this aspect.

Finally, given the difficulties and cost associated with cellular therapies, a system capable of inducing Treg *in vivo* would be ideally suited for treating autoimmunity and transplant rejection. To this end, it would be necessary to use formulations that deliver a combination of Treg inducing factors in a local and sustained fashion *in vivo*, which can be achieved using controlled release formulations that have been developed in the past [156, 203]. The preferred combination of factors to be used *in vivo* might be IL-2, TGF-β, and rapa, as (i) rapa is currently approved for use clinically, (ii) rapa has the ability to suppress a variety of immune functions apart from inducing Treg, and (iii) rapa might be safer than RA, given the latter's ability to induce hypervitaminosis A.

5.0 CONTROLLED RELEASE FORMULATIONS FOR INDUCING REGULATORY T CELLS

5.1 INTRODUCTION

As discussed in Chapter 1 (Section 1.10), increased presence of Treg has been suggested to have positive outcomes in autoimmunity [126, 204], transplant rejection [178, 183] and inflammatory diseases such as dermatitis [205], psoriasis [206-207] and periodontitis [208-209]. Given this evidence, it is not hard to perceive that strategies to boost local Treg numbers could be developed into potential therapeutics to treat these diseases.

Enhancing numbers of Treg at local tissue sites can be achieved by (i) *ex vivo* expansion of Treg followed by their local administration or systemic re-infusion, or (ii) *in vivo* manipulation of immune cells in order to tip the balance between Treg and effector T cells towards Treg. The latter approach is preferable given the stringency associated with *ex vivo* culture of human cells under Good Manufacturing Practice (GMP) conditions [138, 140, 184]. One possible means to achieve increased number of Treg *in vivo* is the use of biologic therapies that selectively enhance Treg numbers and function. Various antibodies (Abs), such as anti- IL-2 monoclonal (m) Ab [210], superagonistic anti-CD28 antibody [211], and agonistic anti-CD4 antibody [212], have been used in the past to increase *in vivo* Treg numbers. However, their

exact mechanism of action has still not been characterized, and their safety in humans remains questionable. In fact, phase I clinical trials of the superagonistic anti-CD28 Ab (TGN1412) resulted in severe negative reactions (cytokine 'storm') in all 6 human subjects who received the Ab [213].

An alternative approach to increase Treg numbers *in vivo* is through the establishment of a local immunosuppressive environment that selectively favors Treg expansion. In Chapter 4 we provide evidence that an environment rich in IL-2, transforming growth factor- β1 (TGF-β) and rapamycin (rapa), favors Treg development even under inflammatory conditions, as suggested by others [196-198]. However, providing a continuous presence of these factors *in vivo*, has proven difficult. Controlled release vehicles for these factors offer a potential solution to these problems.

In this chapter we describe the development and testing of controlled release formulations for IL-2, TGF-β and rapa. We show that a combination of these formulations (called FactorMP henceforth) is capable of *in vitro* Treg induction using either mouse or human cells. Further, we demonstrate that the FactorMP-induced Treg maintain their proliferative capacity and functional capability *in vitro* and express phenotypic surface markers that are consistent with soluble factor induced Treg.

5.2 MATERIALS AND METHODS:

5.2.1 Mice

Six-eight week old C57Bl/6 (B6) and B6.SJL-Ptprca/BoyAiTac (CD45.1) were purchased from Taconic and used within two months. All animals were maintained under

specific pathogen free conditions. Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use for Laboratory Animals and under Institutional Animal Care and Use Committee-approved protocols.

5.2.2 Microparticle Preparation

IL-2 and TGF-β microparticles (IL2MP and TGFβMP, respectively) were prepared using the double emulsion-evaporation technique, as described [156, 203]. For the IL-2MP the following conditions were used. Five µg of recombinant (r) mouse IL-2 (R&D Systems Minneapolis, MN) was mixed with 2 mg of BSA and 5 mM NaCl in 200 µl of de-ionized water. This solution was added to 4 ml of dichloromethane containing 200 mg of poly lactic-co-glycolic acid (PLGA; RG502H, Boehringer Ingelheim Chemicals Inc., Petersburg, VA), and the mixture was agitated using a sonicator (Vibra-Cell, Newton, CT) at 25% amplitude for 10 sec, creating the primary emulsion. This emulsion was then mixed with 60 ml of 2% polyvinyl-alcohol (PVA, MW ~25,000, 98% hydrolyzed; Polysciences) under homogenization (L4RT-A, Silverson, procured through Fisher Scientific) at 3000 rpm for 1 min, creating the second emulsion. The resulting double-emulsion was then added to 80 ml of 1% PVA, and left for 3 hr spinning at 600 rpm. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 80 mTorr).

For the TGFβMP the following conditions were used. One µg of r-human TGF-β (CHO cell-derived, PeproTech, Rocky Hill, NJ) was mixed with 10 mg D-mannitol, 1 mg of BSA, and 15 mM NaCl in 200 µl of de-ionized water. This solution was added to 4 ml of dichloromethane

containing 200 mg of PLGA (RG502H), and the mixture agitated using a sonicator at 25% amplitude for 10 sec, creating the primary emulsion. This emulsion was then mixed with 60 ml of 2% PVA (containing 125 mM NaCl) under homogenization at 3000 rpm for 1 min, creating the second emulsion. The resulting double emulsion was then added to 80 ml of 1% PVA (containing 125 mM NaCl), and left for 3 hr spinning at 600 rpm. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

The rapaMP were prepared using the single emulsion-evaporation technique as described [59, 214]. Briefly, 1 mg of rapa (LC labs, Woburn, MA) dissolved in DMSO was mixed with 4 ml of dichloromethane containing 200 mg of PLGA (RG502H). This solution was mixed with 60 ml of 2% PVA under homogenization at 3000 rpm for 1 min creating the microparticle emulsion. The resulting emulsion was then added to 80 ml of 1% PVA and left for 3 hours spinning at 600 rpm. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

5.2.3 Release Assays

Release assays were conducted by incubating a suspension of particles; (i) 10 mg in 1 ml of media for IL-2MP and TGFβMP, and (ii) 10 mg in 1 ml of PBS (containing 0.2% Tween-80) for rapaMP, on a roto-shaker at 37 °C. At regular time intervals, particle suspensions were centrifuged (250g, 5min), the supernatant removed, and the particles re-suspended in 1 ml of appropriate solution. The amount of each cytokine in the supernatant was measured using a cytokine-specific ELISA (R&D systems, Minneapolis, MN), and the amount of rapa was measured using spectrophotometry (absorbance at 278 nm).

5.2.4 Mouse T cell isolation

Spleen and lymph nodes were dissected from B6 or CD45.1 mice. Following mechanical digestion, the tissue suspension was passed through a 70 μm nylon filter to obtain a single cell suspension of leukocytes. Predominantly naïve $CD4^+$ T cells were isolated from this suspension with a $CD4^+$ T cell negative isolation kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. These purified $CD4^+$ T cells were used in cell culture and suppression assays.

5.2.5 Induction of regulatory T cells (Treg)

For Treg induction experiments, naïve T cells were cultured either in direct contact with FactorMP (in 96-well round bottom cell culture plates), or separated from FactorMP by permeable transwell inserts (HTS Transwell®-96, 0.4μm pore size; Corning, Lowell, MA). Dynabeads® mouse T-activator CD3/CD28 beads (Dynabeads®; Invitrogen, Carlsbad, CA) were used at a 2:1 (beads:T cells) ratio to activate T cells, and cultures maintained for 4 days. For cultures in the presence of soluble factors, the following concentrations of factors were used: 10 ng/ml IL-2, 5 ng/ml TGF-β and 10 ng/ml rapa (corresponds to a total amount of 2 ng IL-2, 1 ng TGF-β and 2 ng rapa). The following quantities of microparticles were used for the induction experiments in 200 μl of cell culture media: 2 mg TGFβMP, 0.5 mg IL-2MP, and ~ 0.01 - 0.05 mg rapaMP. To determine the phenotype of cells after culture, cells were stained with anti-CD4 (L3T4), anti-FoxP3 (FJK-16s), anti-CD25 (PC61.5), anti-glucocorticoid-induced TNFR-induced protein (GITR; DTA-1), anti-folate receptor-4 (FR4; eBio12A5) (antibodies from eBiosciences,

San Diego, CA) and anti-cytotoxic T-lymphocyte antigen 4 (CTLA4; UC10-4B9, from Biolegend, San Diego, CA). To determine iTreg proliferation, naïve T cells were stained with carboxyfluorescein diacetate succinimidyl ester cell tracer (CFSE; Invitrogen, Carlsbad, CA) prior to activation with Dynabeads®. Stained cells were then analyzed on a BD-LSRII flow cytometer. For suppression assays, cells (from Transwell® cultures) were collected after 4 days of culture and used as described below.

5.2.6 Suppression assay

Freshly isolated naïve $CD4^+CD45.1^+$ T cells were stained with CFSE (Invitrogen, as per manufacturer's instructions) and co-cultured with induced Treg (generated as described above) at different ratios in 96-well plates. The number of naïve $CD4^+CD45.1^+$ cells was kept constant at 50,000 cells / well. For stimulation, 50,000 Dynabeads® per well were used. Co-cultures were carried out for 4 days, after which cells were stained for flow cytometry.

5.2.7 Human T cell culture

$CD4^+$ T cells were isolated from human PBMC using the CD4 negative isolation kit (Miltenyi Biotec, Auburn, CA). Cells (500,000) were cultured in 0.5 ml of AIM-V cell culture media (supplemented with serum) in the presence of human T cell activation beads (anti-CD2, anti-CD3 and anti-CD28 coated beads, Miltenyi Biotec, Auburn, CA). Cell culture media was supplemented with additional factors or FactorMP at the following concentrations: 500 U/ml recombinant-human IL-2, 10 ng/ml TGF-β, 2 ng/ml rapa, 8 mg/ml of TGFβMP, and/or 0.02 mg/ml of rapaMP. Following 4 days of culture, cells were collected, stained with anti-human

CD3 (BD Pharmingen), anti-human CD4 (L200, BD Pharmingen), anti-human CD25 (BC96, BD Pharmingen), anti-human FoxP3 (PCH101, BD Pharmingen) and Treg induction analyzed by flow cytometry. Soluble IL-2 was used in all of these cultures instead of IL-2MP, as IL-2MP encapsulated recombinant mouse IL-2 and not the human protein.

5.3 RESULTS

5.3.1 Microparticle Characterization

IL2MP, TGFβMP, and rapaMP were all prepared under similar conditions, using the same polymer (PLGA, RG502H, viscosity 0.16-0.24 dl/g). Scanning electron micrographs (Figure 25, top panel) show that individual particles are spherical and confirm the volume average size distributions (IL2MP = 25.5 ± 7.5 μm; TGFβMP = 16.7 ± 6.3 μm; rapaMP = 16.7 ± 6.4 μm; errors indicate standard deviation from the mean for each particle set). Additionally, the images show that IL2MP have slightly porous exterior surfaces. These particles were specifically formulated to be porous (by altering osmotic pressures between the inner emulsion and the outside aqueous phase during microparticle preparation) so that a high initial burst followed by continuous release could be obtained (Figure 1, bottom panel) [159-160]. Further, we observe a linear release of TGF-β following a ~2 week lag phase, and a continuous release from rapaMP (Figure 25, bottom panel).

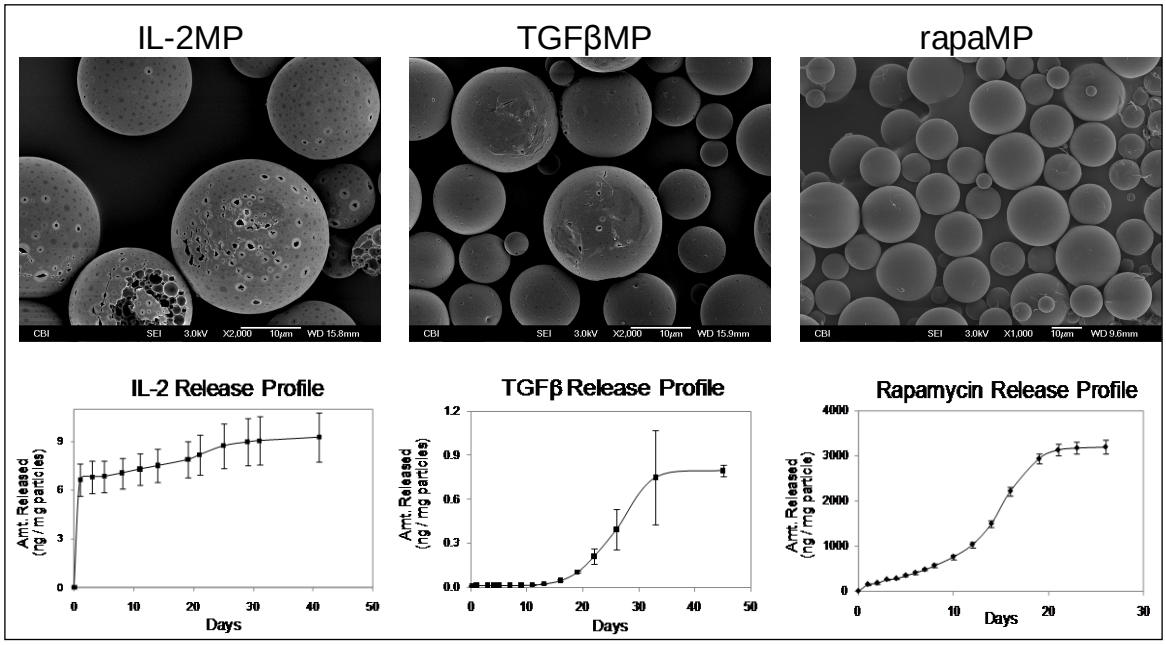

Figure 25: Microparticle Characteristics.

Scanning electron micrographs (top panel) and in vitro release profiles (bottom panel) of IL-2, TGFβ (in cell culture media) and rapa (in saline containing 0.2% Tween-80). Error bars on release profiles represent standard deviation based on n = 6 measurements for IL-2MP and TGFβMP, and n= 3 measurements for rapaMP.

5.3.2 Treg Induction

Soluble IL-2, TGF-β and rapa have been shown previously to induce Treg (iTreg) [196-197]. We wanted to determine if degradable polymer-based formulations designed to sustain the release of these factors could induce Treg reliably. Indeed, we observed that the microparticle formulations were similar to soluble factors in their *in vitro* Treg induction efficacy, as measured by FoxP3 expression (Figure 26A and 26B). In addition, the FactorMP were capable of inducing Treg by releasing equivalent (10-15 ng/ml IL-2 and 10-50 ng/ml rapa), or reduced (1-2 ng/ml of TGF-β)

total amounts of the factors over 4 days of culture. Further, we observed that iTreg were capable of robust proliferation (as observed through the dilution of the CFSE stain, Figure 26A).

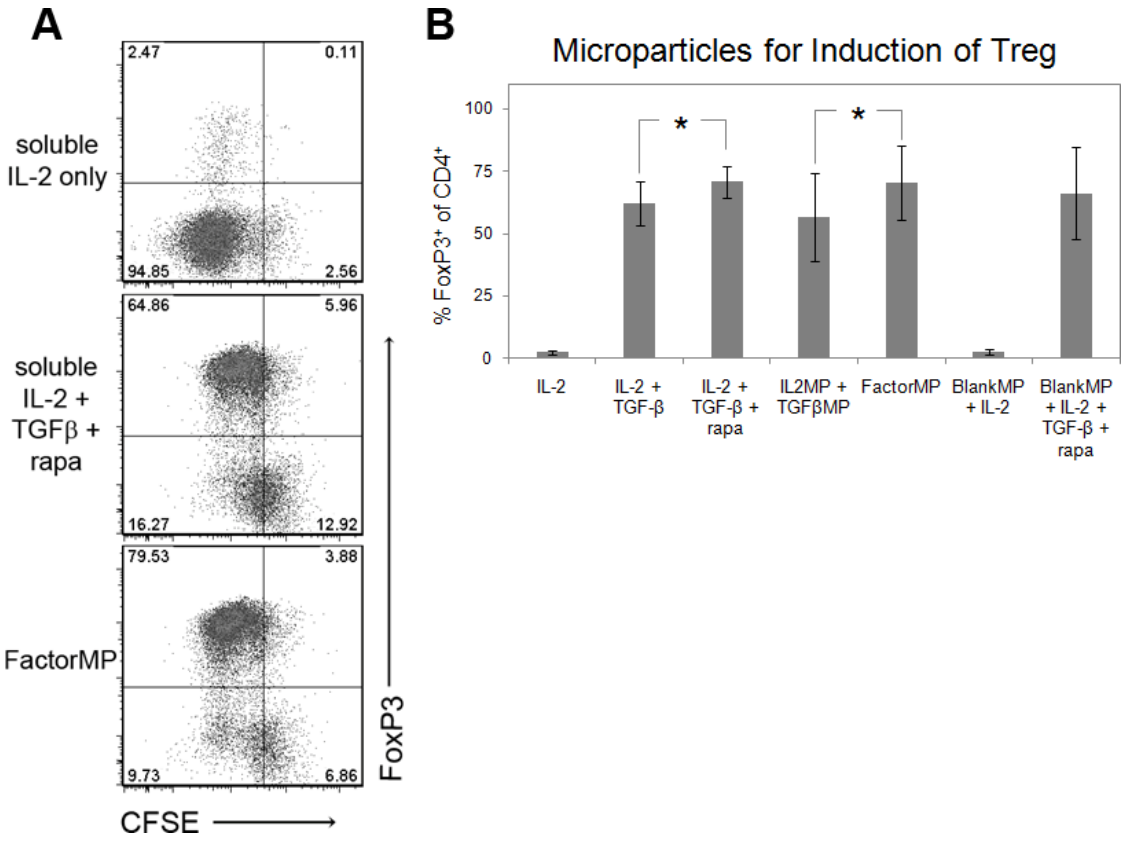

Figure 26: FactorMP induce mouse Treg.

A – representative flow cytometry dot plots (gated on CD4-expressing cells) of naïve T cells stimulated in the presence of soluble factors or FactorMP. The X axis on these plots represents CFSE, which is a cell proliferation marker and the Y axis represents intracellular FoxP3, which is a definitive marker for mouse Treg. B – quantitative analysis of the percentage of CD4+ T cells that express FoxP3 after culture for 4 days under different conditions; * indicates $p<0.05$ based on $n \geq 3$ independent experiments.

5.3.3 Phenotype and function of microparticle induced Treg phenotype

In addition to FoxP3, Treg are known to express many other characteristic surface proteins. We tested for 4 canonical surface markers: CD25, FR-4 and GITR. CD25 is the high-affinity IL-2 receptor, which increases sensitivity to IL-2 and is important for Treg proliferation. FR-4, a folate receptor, is required for folic acid sensing and uptake, which in turn prolongs Treg survival. Finally, GITR (glucocorticoid-induced TNF receptor-related protein) is a surface receptor that has been suggested to play an important role in Treg survival and suppression. We observed that FactorMP-iTreg expressed these surface markers at levels equivalent to those on soluble factor-iTreg (Figure 27). Importantly, although the expression of these surface proteins, along with FoxP3, suggests that these cells are Treg, it does not guarantee suppressive function. In order to test the ability of FactorMP-iTreg to suppress naïve T cell proliferation, we adopted an *in vitro* co-culture system described previously [215]. In these co-culture suppression assays, we observe that FactorMP-iTreg indeed possessed suppressive capabilities similar to Treg induced by soluble factors and natural Treg (Figure 28).

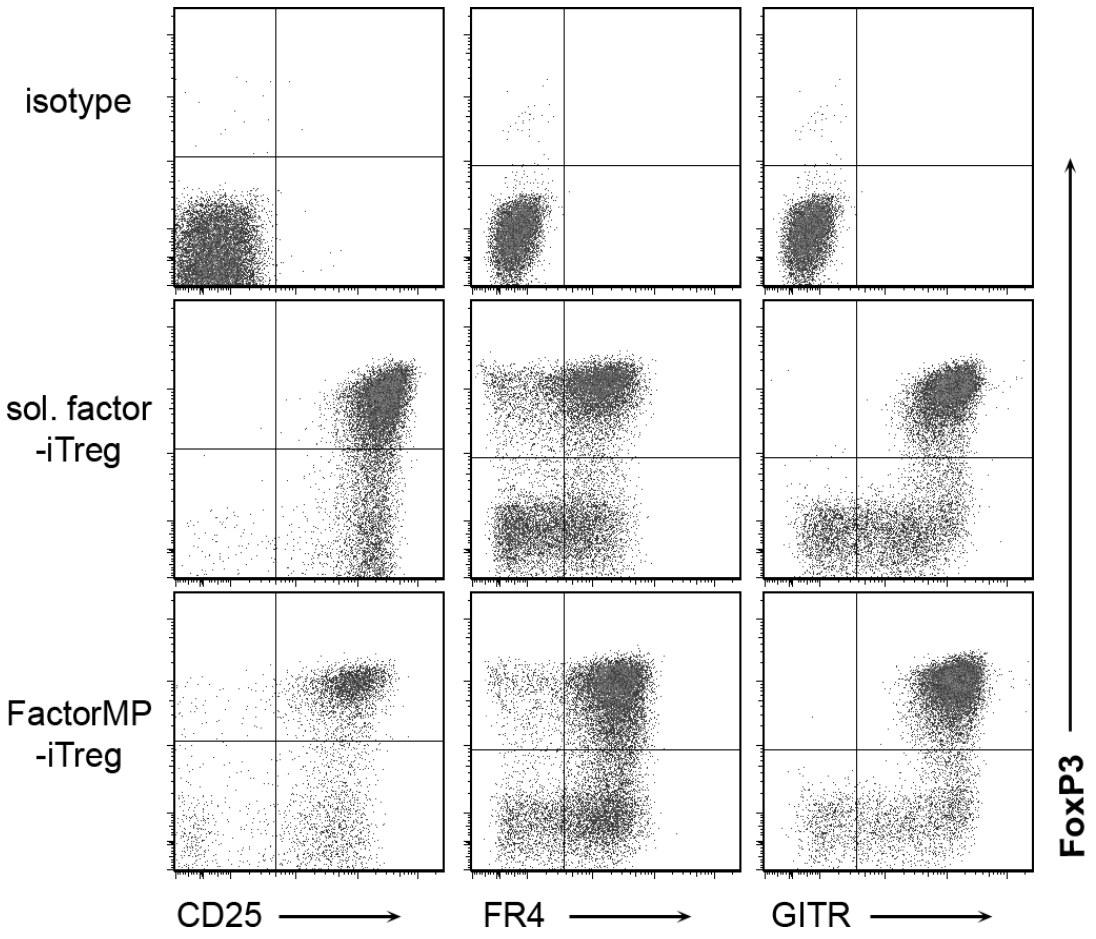

Figure 27: FactorMP-iTreg express canonical Treg surface markers.

Representative flow cytometry dot plots (gated on CD4 expressing cells) showing the expression of surface markers and intracellular FoxP3 on naïve T cells stimulated in the presence of soluble factors or FactorMP.

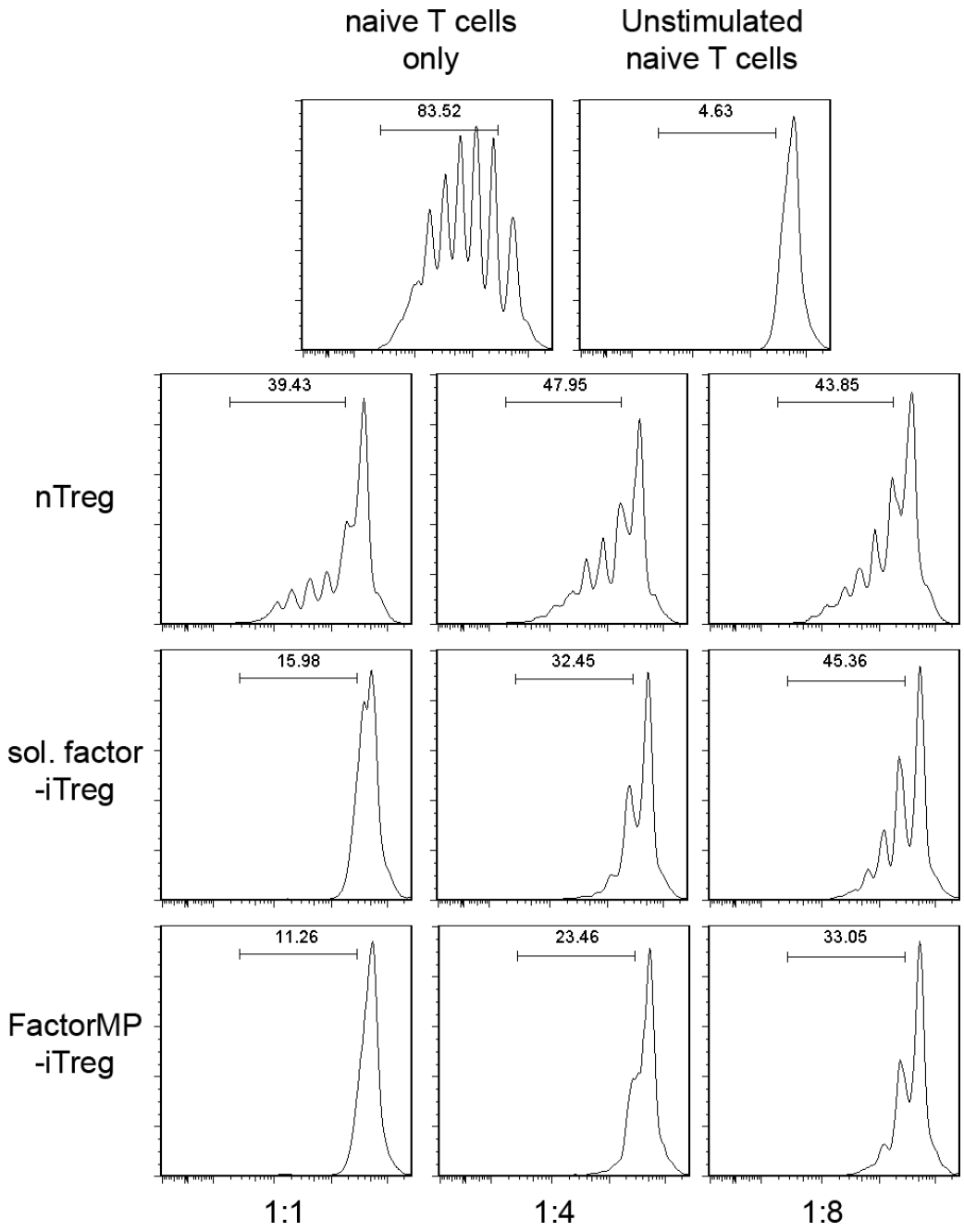

Figure 28: FactorMP iTreg are suppressive.

Representative plots of CFSE dilution showing that the FactorMP-iTreg can suppress naïve T cell proliferation. Gates on individual plots indicate the percentage of proliferating cells. Ratios indicate the number of Treg in culture to the number of naïve T cells.

5.3.4 Microparticle formulations can induce Treg from human T cells

Human T cells isolated from PBMC can also be induced to a Treg phenotype using soluble IL-2, TGF-β and rapa [216-217]. For potential clinical application of our technology, we needed to determine if the microparticle formulations were capable of inducing Treg from human T cells. To this end, human T cells were cultured in the presence of soluble factors or FactorMP. We observe that the microparticles were equally capable of inducing Treg when compared to the soluble factors, while releasing equivalent (1-10 ng/ml rapa) or reduced (2-4 ng/ml TGF-β) amounts of factors (Figure 29A and 29B).

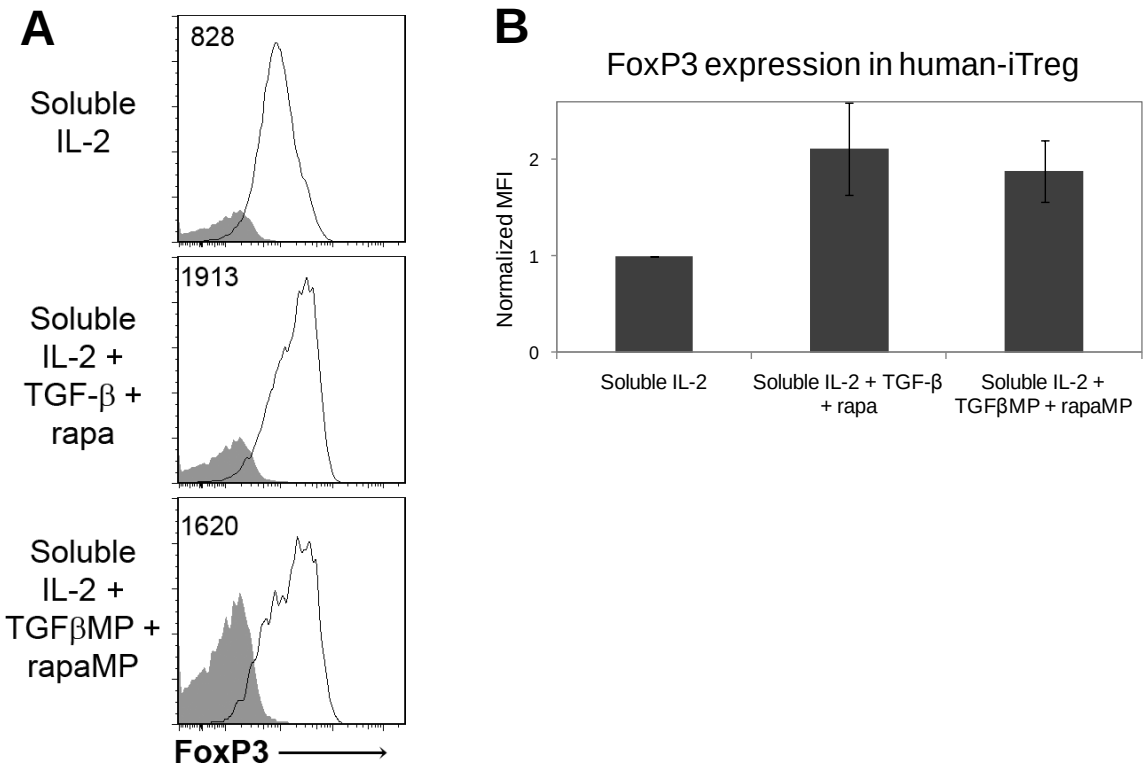

Figure 29: Microparticle formulations can generate human-iTreg.

A – representative plots displaying FoxP3 expression profile on human T cells cultured under different conditions. Numbers in plots represent the median fluorescence intensities (MFI). **B** – quantitative analysis of normalized FoxP3 MFI as determined from 2 independent experiments (n≥3). MFI was normalized by determining the ratio of experimental MFI and control (soluble IL-2 treated cells) MFI.

5.4 DISCUSSION

Therapies that enhance Treg numbers and function have the potential to cure diseases associated with adverse inflammation [126, 129]. Indeed, clinical trials are currently testing cellular therapies involving Treg as potential therapeutics for treating graft versus host disease [138]. However, Treg-based cellular therapies face many challenges, which include, but are not limited to: (i) difficulties in isolating pure and homogenous populations and large quantities of Treg

from the blood, (ii) inconsistent maintenance of Treg phenotype and suppressive function post-proliferation, and (iii) the need for GMP facilities [138, 140, 184]. Hence, acellular therapies that can increase numbers and/or suppressive potency of Treg without the need for *ex vivo* culture could be transformative.

One potential method to increase the ratio of Treg to effector T cells is to establish an environment rich in IL-2, TGF-β and rapa as described [196-198]. Such an immunosuppressive, Treg-inducing environment can be attained through the sustained release of these factors at a local site. To that end, release formulations were fabricated using an FDA approved, widely used polymer, PLGA. These formulations were specifically prepared to ensure that the release profiles fit our needs for the induction and proliferation of Treg. For example, IL-2 is required for Treg survival, and it has been suggested high initial doses might help Treg grow better and resist apoptosis [218-219]. Hence, we prepared slightly porous IL2MP that have a high initial burst followed by a slow continuous release of the factor over a 5 week time frame (Figure 25). We also needed a continuous release formulation for TGF-β, which has not been fabricated previously. Accordingly, we prepared new TGF-β formulations capable of linear release over a 3 week period (Figure 25) beginning after a 2 week lag phase. Although this 2 week initial lag phase was unexpected, unpublished data (Hwang M.P. et. al., manuscript in preparation) from our lab suggests that the initial lag phase could be due to ionic interactions between TGF-β and PLGA. This ionic interaction could be a result of the high isoelectric point (pI) of TGF-β and the relatively low pH conditions inside the microparticles. Regardless, to overcome the problem of the initial lag phase we simply pre-incubated the TGFβMP (18-22 days) prior to use in cell culture. Finally, rapaMP were also formulated to be similar in size to the IL2MP and TGFβMP

and release continuously over a 2-3 week time frame as previously demonstrated (Figure 25) [59, 214].

Importantly, the combination of these microparticle formulations (FactorMP) appears to be as effective as soluble factors at inducing Treg from naïve T cells in *in vitro* cultures. Additionally, we determined that the FactorMP iTreg were capable of robust proliferation (Figure 26), expressed canonical surface markers representative of Treg (Figure 27), and were able to suppress naïve T cell proliferation in an *in vitro* suppression assay (Figure 28). Further, it was observed that Treg induction and proliferation occurred even when the cells were in contact with microparticles, suggesting that the microparticles do not have any adverse on these cells. Finally, we observed that these microparticles are equally effective at inducing human Treg. The human-iTreg showed high expression of FoxP3 and were also capable of proliferation (Figure 29). Overall, our data suggests that these FactorMP have the potential to be used *in vivo* for local Treg induction at sites of transplant rejection or autoimmunity.

We envisage these particulate formulations could be used as an ‘off-the-shelf’ therapeutic for creating an immunosuppressive environment and increasing the presence of Treg at sites of inflammation. We are currently testing these particles in *in vivo* mouse models of contact dermatitis and periodontitis, diseases where immune homeostasis is lost and needs to be restored. Another possible application for such formulations would be skin and composite tissue transplantation, where a local immunosuppressive environment could prevent graft rejection while retaining the integrity of the systemic immune system that can continue to fight infections and inhibit malignancies.

6.0 CONCLUSIONS AND FUTURE WORK

Synthetic systems that modulate regulatory immune responses *in vivo* hold tremendous potential for the treatment of autoimmunity, transplant rejection and other inflammatory diseases such as periodontitis and dermatitis. We describe three different degradable and biocompatible controlled release formulations that can possibly be used to modulate the immune system *in vivo*.

One of the formulations involves delivery of immuno-modulatory agents to DC. Immunosuppressive drugs such as rapa are capable of altering DC function [30-32], and our data shows that intracellular DC-specific delivery of rapa using controlled release formulations (rapaMP) can generate suppressive DC that are significantly better than soluble rapa-treated DC at suppressing T cell proliferation (Chapter 2). This altered DC function could possibly be due to the greatly lowered expression of co-stimulatory proteins on the surface of DC that have internalized rapaMP. A system capable of DC-specific delivery of immunosuppressive agents can potentially be used to suppress the stimulatory functions of DC in inflammatory bowel disease, type I diabetes and even skin transplant rejection.

The second formulation described here is based on mimicking a strategy employed by tumors to evade the immune system. Tumors and tumor-associated cells are known to secrete a chemokine, CCL22, for the recruitment of regulatory T cells (Treg) and consequent evasion of immune responses [173-174]. We synthesized a continuous and sustained release formulation of

CCL22 using PLGA microparticles (CCL22MP), to mimic tumor cells. We show that CCL22MP are capable of recruiting Treg using a new, non-invasive *in vivo* migration assay (Chapter 3). Additionally, injection of CCL22MP at sites of cell (allogeneic LLC transplant studies) or composite tissue (alogeneic whole limb transplant studies) transplants leads to a considerable delay in rejection of the graft, suggesting the potential of these formulations to be used to treat transplant rejection as well as autoimmunity.

Further, we have thoroughly characterized a combination of factors that can induce Treg from naïve T cells and help to establish a local immunosuppressive environment *in vivo*. Previously, the combination of IL-2, TGF-β and all-trans retinoic acid (RA) and a combination of IL-2, TGF-β and rapa have shown to induce Treg (iTreg) *in vitro* [189-190, 196-197]. While the phenotype and functional capabilities of iTreg generated in the presence of IL-2, TGF-β and RA (RA-iTreg) have been thoroughly characterized, the characteristics of iTreg generated in the presence of IL-2, TGF-β and rapa (rapa-iTreg) are yet to be determined. Our studies show that rapa-iTreg do not differ from RA-iTreg in their *in vitro* and *in vivo* suppressive capabilities and appear to be stable under long-term culture conditions *in vitro*. However, a major difference between these iTreg was their *in vivo* migratory capacity, with rapa-iTreg retaining the capacity to migrate to lymphoid tissues throughout the body, while RA-iTreg migrated primarily to the gut (Chapter 4). Given this observation and the fact that rapa helps to maintain a suppressive environment (while RA need not), we chose the combination of IL-2, TGF-β and rapa to fabricate controlled release vehicles that can potentially be used *in vivo*. We show that controlled release formulations of this combination of agents (FactorMP) can induce and promote Treg proliferation from both mouse and human naïve T cells (Chapter 5).

Finally, the synthetic systems developed herein, may be used individually or in combination to modulate immune responses *in vivo*, with potential applications in treating autoimmunity and transplant rejection. Furthermore, these systems are specifically designed as modular platforms that can easily be modified to incorporate a protein or drug of choice, for the development of novel therapeutics as well as a tool to study cellular responses *in vitro* and *in vivo*.

APPENDIX A

CCL22MP IN COMPOSITE TISSUE TRANSPLANTATION

In chapter 3, we demonstrate that CCL22MP are capable of recruiting Treg *in vivo* and delaying rejection of allogeneic cellular transplants. Next, we wanted to determine if CCL22MP could prolong graft survival in a clinically relevant model for transplantation. Whole limb transplantation in rats has previously been established as a good pre-clinical model for composite tissue transplantation [220-221]. In this section, we test the ability of CCL22MP to delay graft rejection in this pre-clinical model of transplantation.

A.1 METHODS

Whole limb transplants were performed as described (Figure 30) [220-221]. Briefly, the hind limbs of Brown-Norway rats were transplanted to Lewis rats (completely mismatched tissue). Four days prior and 1 day post transplantation the host rats received anti-lymphocyte serum treatment. On the day of transplantation, the rats received a sub-cutaneous injection of 20 mg of CCL22MP (or BlankMP as control). Following transplantation, the host rats received 0.5 mg/kg FK506 daily for 20 days. On day 18 post transplantation, a second dose of CCL22MP was

injected sub-cutaneously in one treatment group. The transplants were monitored daily for signs of rejection as described [222].

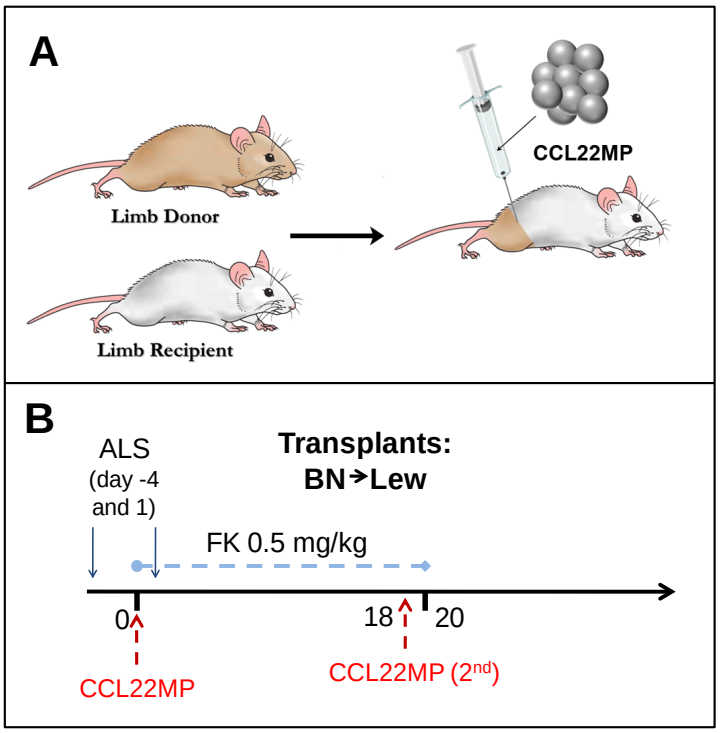

Figure 30: Composite tissue transplantation in rats.

A – cartoon depicting the use of CCL22MP in an allogeneic whole limb transplant model. **B** – schematic describing the immunosuppressive regimen used to delay graft rejection. ALS indicates anti-lymphocyte serum , FK indicates FK-506 (tacrolimus), BN indicates Brown Norway rats and LEW indicates Lewis rats.

A.2 RESULTS AND DISCUSSION

Hautz et.al. [222] have demonstrated that cessation of systemic immunosuppression leads to rapid rejection of the transplanted limb. We observe the same in our experiments, where all but one of the BlankMP treated transplants rejected by day 50 post transplantation (systemic FK506 treatment was stopped at day 20 post transplantation as described in Figure 30). We observe that

a single dose of CCL22MP does not prolong transplant survival with all the transplants rejecting within 50 days post transplantation. However, transplants that received the double dose of CCL22MP show enhanced survival rates ($p = 0.1$) with 2 out of 13 transplants surviving over day 100 (Figure 31A). Additionally, we observe that most of the CCL22MP (double dose) treated transplants have an intact epidermal layer with low infiltration of mono-nuclear cells, while a majority of the BlankMP treated transplants have very high infiltration of mono-nuclear cells and show loss of the epidermal layer 25 days post transplant (Figure 31B).

Currently, additional transplant experiments are underway to determine the dosing schedule and the concentration of CCL22MP required for observing significant differences in transplant survival rates. This preliminary study demonstrates the promise of CCL22MP in delaying transplant rejection, and its potential for translation to the clinic.

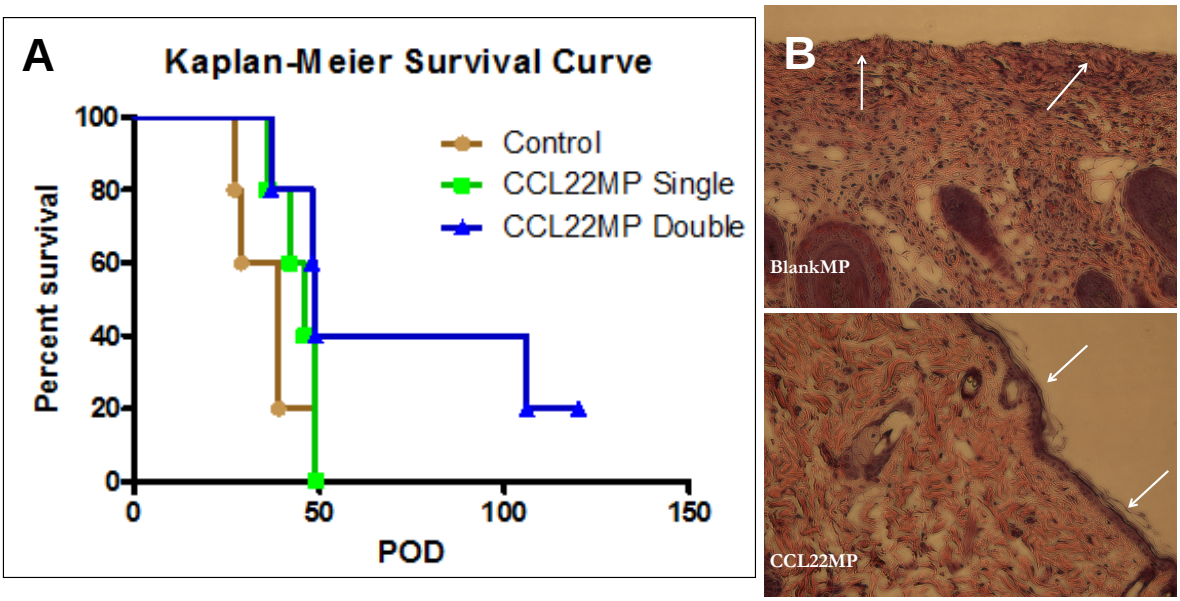

Figure 31: CCL22MP can delay composite tissue graft rejection.

A – survival curves illustrating that the double dose of CCL22MP is better at delaying transplant rejection when compared to the control and single CCL22MP dose groups ($p \sim 0.1$). B – hematoxylin and eosin staining of skin biopsies from the transplanted hind limb 25 days post transplantation. White arrows point to the epidermal layer, which is present in the CCL22MP (double dose) injected group but absent in the BlankMP group.

APPENDIX B

SURFACE LABELING OF PLGA MICROPARTICLES WITH ANTI-CD3/ANTI-CD28 TO MIMIC DC FUNCTION

As discussed in chapter 1, the primary function of DC is to activate naïve T cells through antigen presentation (Signal 1) and co-stimulation (Signal 2), which is achieved through surface receptor-receptor interaction. Synthetic systems that hope to mimic DC function must be able to replicate this function reliably. The first completely artificial DC systems developed specifically for the purpose of activating and expanding naïve T cells was the Dynabeads® technology (from Dynal Biotech., now a part of Life Technologies). In this system, magnetic epoxy beads are labeled on the surface with antibodies against CD3 and CD28 (receptors that are part of the TCR and co-stimulatory apparatus on T cells, respectively). Although this system can successfully activate and expand naïve T cells *in vitro*, it cannot be used *in vivo* as the beads are made of a non-degradable, non-biocompatible material. A possible solution to this problem is to replace the epoxy beads with degradable, biocompatible particles like those made of PLGA. Herein, we describe a strategy for labeling the surface of PLGA microparticles with antibodies for naïve T cell stimulation.

B.1 PROTOCOL FOR LABELING THE SURFACE OF PLGA PARTICLES

Labeling of the surface of PLGA particles is achieved by covalently linking free amine groups on the streptavidin to the free COOH (end groups on the polymer) groups on the surface of particles, followed by the addition of biotinylated antibodies (as a free COOH group is required on the surface, particles fabricated with end-capped polymers cannot be labeled using this procedure).

i. Weigh out 10 mg of PLGA particles in 1.5 ml Lo-Bind Eppendorf tubes.
ii. Wash particles with 1 ml of **bicarbonate buffer** (pH 9.6-9.8) – twice.
iii. Wash particles with 1 ml of **MES buffer** (pH 6) – twice.
iv. Add 4mM **EDC** (Acros) and 5mM **sulfo-NHS** (Pierce). This can be done as below –
    a. Make up a 1.91 mg/ml solution of EDC in MES buffer. Add 400 ul of this to particles
    b. Make up a 2.17 mg/ml solution of sulfo-NHS in MES buffer and add 500 ul of this.
    c. This can be scaled up depending on the number of particle sets.
v. Make up the final volume to 1 ml using MES buffer and to control groups do not add these chemicals (add only 1 ml of MES buffer).
vi. Allow for chemical reaction to take place for 1 hour on roto-shaker at room temperature.
vii. Wash particles once with **DPBS**.
viii. Add 100 ul of **streptavidin solution** (1 mg/ml stock) and make up final volume to 1 ml using DPBS.
ix. Allow to react for 4 hours on roto-shaker at room temperature.

x. Wash 2 times with DPBS.

xi. Wash once with **1% BSA** prepared in DPBS.

xii. Add 1 ml of DPBS to particles, followed by appropriate amounts of **anti-CD3** and **anti-CD28** (amounts depend on the size of the particles and depends on the ratio of the 2 antibodies desired).

xiii. Allow to react for 90 min at room temperature on roto-shaker.

xiv. Wash 3 times with DPBS.

xv. Wash once with D.I. water

xvi. Add 100 ul of D.I. water and freeze dry for 48 hours.

Solutions and reagents required:

i. **Bicarbonate Buffer (pH 9.6-9.8):** Add 1.68 grams of Sodium Bicarbonate to 400 ml of D.I. water, followed by 1 ml of 4N NaOH. The resultant solution is biocarbonate buffer at a pH of around 9.6-9.8.

ii. **MES buffer (pH 6):** Add 9.76 grams of MES (2-(N-morpholino) ethanesulphonic acid) to 450 ml of D.I. water. Adjust pH to 6 with 4N NaOH, and make up volume to 500 ml final.

iii. EDC and NHS solutions – make as suggested above.

iv. DPBS – Phosphate buffered saline from Invitrogen

v. Streptavidin solution – Dissolve 1 mg of Streptavidin (Promega) in 1 ml of DPBS. Make 200 ul aliquots and freeze. Add 100 ul of this to each particle set.

vi. 1% BSA – Add 1gram of BSA to 100 ml of DPBS to prepare a 1% BSA solution. Scale down to appropriate amounts for small experiments.

B.2 RESULTS

PLGA microparticles (15-20 μm avg. diameter) were labeled with anti-CD3 (biotin) and anti-CD28 (biotin) using the aforementioned procedure. Thymidine incorporation and CFSE dilution assays (a measure of T cell proliferation) showed that anti-CD3 and anti-CD28 labeled microparticles (aAPC) were capable of activating naïve T cells (Figure 32). Additionally, we observe that a ratio of 2:1 (anti-CD3:anti-CD28) as well a concentration of 4 μg of anti-CD3 worked the best at enabling T cell proliferation (Figure 33).

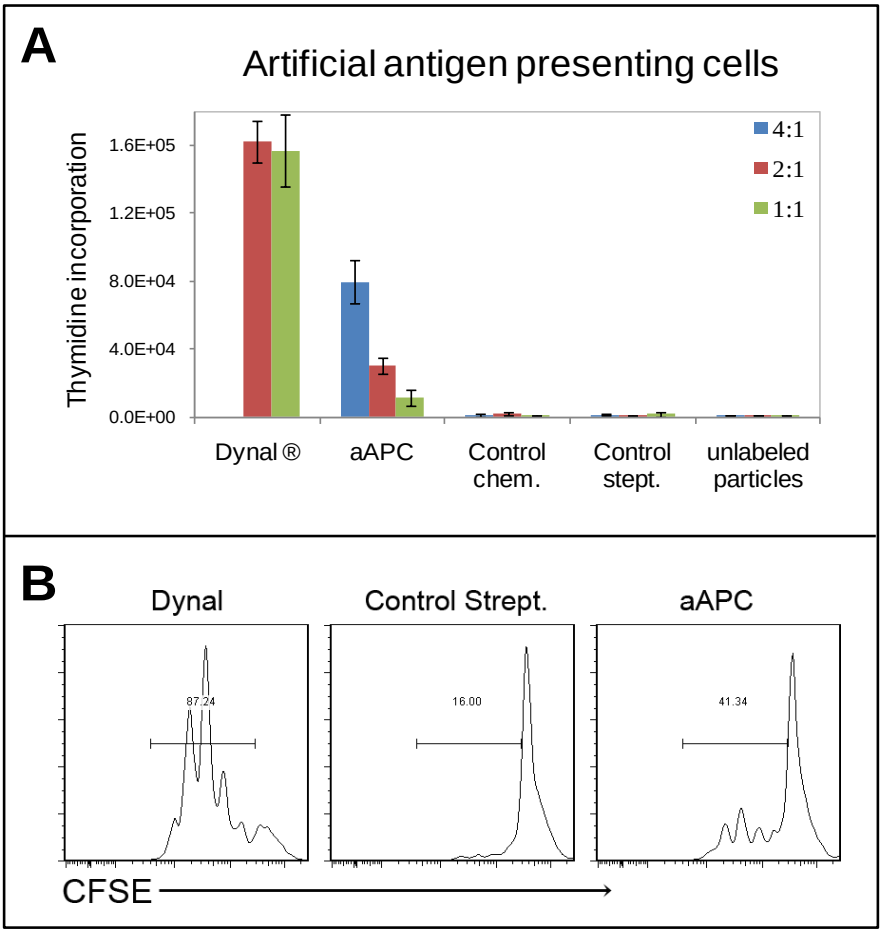

Figure 32: anti-CD3/anti-CD28 labeled particles can function as artificial antigen presenting cells (aAPC).

The ability of dynal ® (positive control) beads and PLGA microparticles (aAPC and negative controls) in proliferating naïve T cells as measured using the thymidine incorporation assay (A) and the CFSE dilution assay (B). Control Strept. refers to microparticles labeled with streptavidin and antibodies in the absence of EDC and NHS (see protocol for labeling).

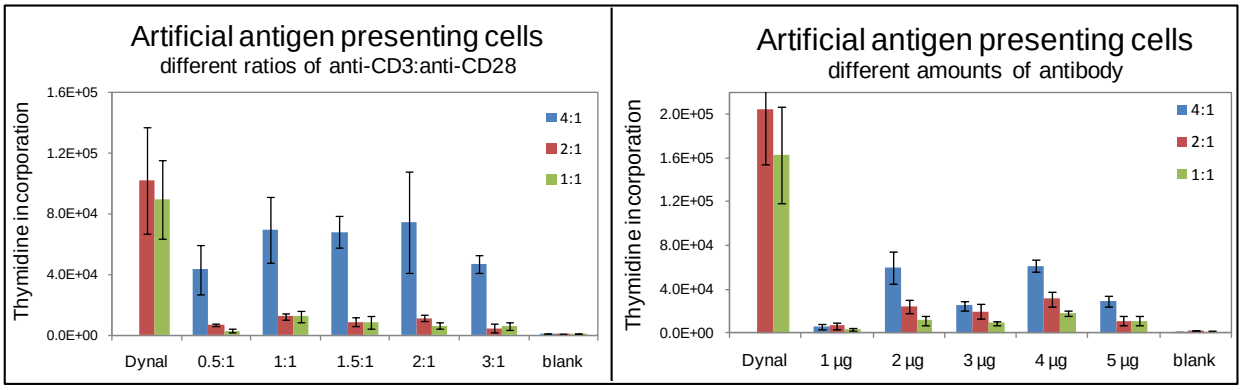

Figure 33: Optimization of the ratio and amount of antibodies on PLGA microparticles to function as aAPC.

Thymidine incorporation assays showing that a ratio of 2:1 (anti-CD3:anti-CD28) and 4 µg of anti-CD3 works the best at stimulating T cell proliferation.

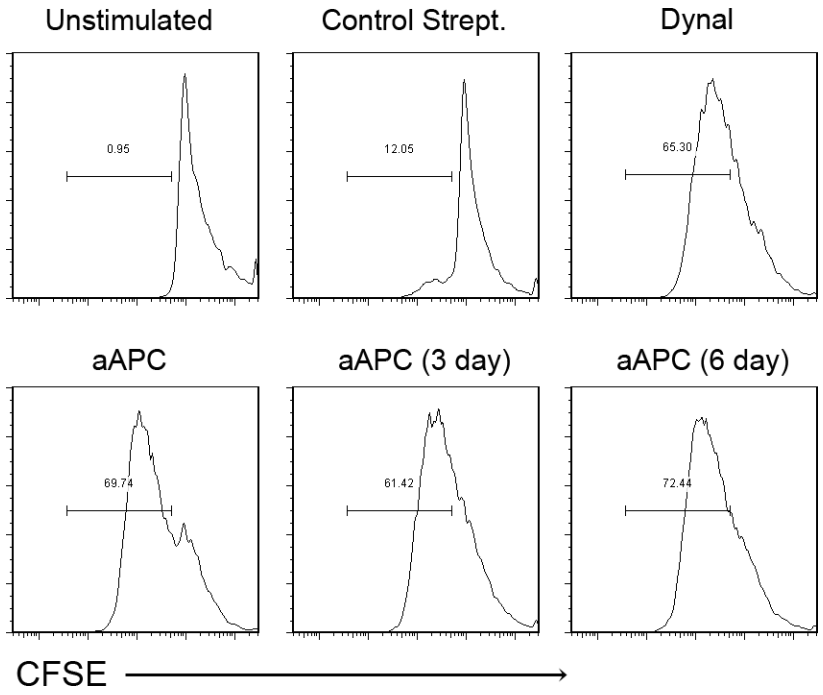

Figure 34: Antibody labeled microparticles remain functional following degradation in saline.

CFSE dilution assay measuring the ability of aAPC to stimulate T cell proliferation following incubation in saline (simulating conditions for degradation).

One of the most common concerns with a procedure that involves covalent coupling of antibodies to the surface of degradable microparticles is that the antibodies would be de-coupled rapidly as the microparticle degrades. However, it is well established that PLGA microparticles degrade from the inside-out (bulk degradation) [223], and hence we presumed that the antibodies would not be detached in a short timeframe. Indeed, we observe that the covalently coupled antibodies remain functionally active on the surface of microparticles even after incubating the particles in saline at 37°C for 3 and 6 days (Figure 34).In conclusion, our data suggests that PLGA microparticles labeled with anti-CD3 and anti-CD28 can successfully mimic DC function of naïve T cell activation.

APPENDIX C

FABRICATING POROUS PARTICLES

C.1 INTRODUCTION

Degradable polymeric microparticles can be classified based on their surface architecture as non-porous (solid) or porous. Porous microparticles are of tremendous interest to controlled release engineers and pharmaceutical scientist for two reasons: (i) they have low aerodynamic diameters that allow for their use in pulmonary delivery applications [224], and (ii) they allow for alteration of release kinetics (by changing the porosity of the particle) from a drug-loaded microparticle without changing other physical parameters of the microparticle (such as polymer molecular weight or geometric diameter of the particle) [225].

Industrially, porous microparticles are commonly fabricated using the spray drying method. However, the spray drying procedure has many drawbacks such as, loss of structural integrity of proteins due to the high temperatures required to produce spray dried particles [226], low total yields of microparticles [227-228], and the presence of residual moisture content leading to protein instability [229]. An alternative to spray drying is the use of an emulsion/evaporation technique in the presence of osmotic gradients to fabricate microparticles

[224-225, 230-231]. Thorough characterization of the conditions required to fabricate microparticles of different porosities using the latter method have not yet been performed.

In this study we present descriptive techniques and a few conditions required for the fabrication of porous microparticles.

C.2 MATERIALS AND METHODS:

C.2.1 Materials

Poly lactic-co-glycolic acid (PLGA, RG502 and RG502H) as well as Poly lactic acid (PLA) was ordered from Boehringer Ingelheim Chemicals, Inc. Polyvinylalcohol (PVA, MW 25,000) was purchased from Polysciences, Inc.

C.2.2 Preparation of Microparticles

Porous microparticles were prepared using the emulsion/evaporation technique [224-225]. Briefly, an aqueous phase (200 µl) consisting of varying sodium chloride concentrations was added to 4 ml of dichloromethane (DCM) containing 200 mg of polymer. This mixture was sonicated 10 seconds (25% amplitude, Silverson L4RT-A), to create the primary emulsion (water in oil). The primary emulsion was then mixed with 60ml of 2% w/v PVA, and homogenized (Vibra-Cell Ultrasonic Processor) for 1 minute at 2,500rpm, creating the second emulsion (oil in water). The resulting solution was combined with 80ml of 1% w/v PVA and allowed to stir for 3

hours at room temperature to allow for DCM to evaporate. Microparticles were then washed using de-ionized water (x4), and lyophilized for 48 hours. The particles were stored at -20 C.

C.2.3 Particle characteristics

Volume impedance measurements on a Beckman Coulter Multisizer III, were used to determine the particle size distribution. Surface characteristics and particle integrity were assessed using scanning electron microscopy (JSM6330F).

C.2.4 Surface Area and Density

Surface area measurements were obtained with the use of nitrogen utilized BET (Quantachrome NOVA 2000 series) surface analyzer. Density measurements were obtained using an Ultrapycnometer.

C.3 RESULTS AND DISCUSSION

In this study, we observe that increasing sodium chloride in the primary emulsion resulted in fabrication of particles with increasing porosity (Figure 35). A possible explanation for the occurrence of these pores could be the osmotic imbalance between the inner emulsion and the outer aqueous phase, which leads a rapid influx of water causing the emulsions to swell and burst (thereby leaving an open pore). Additionally, the formation of pores is not dependent on the type

of excipient (ionic vs. non-ionic) used in the inner emulsion as both sodium chloride and mannitol loaded particles show porous surfaces (Figure 36).

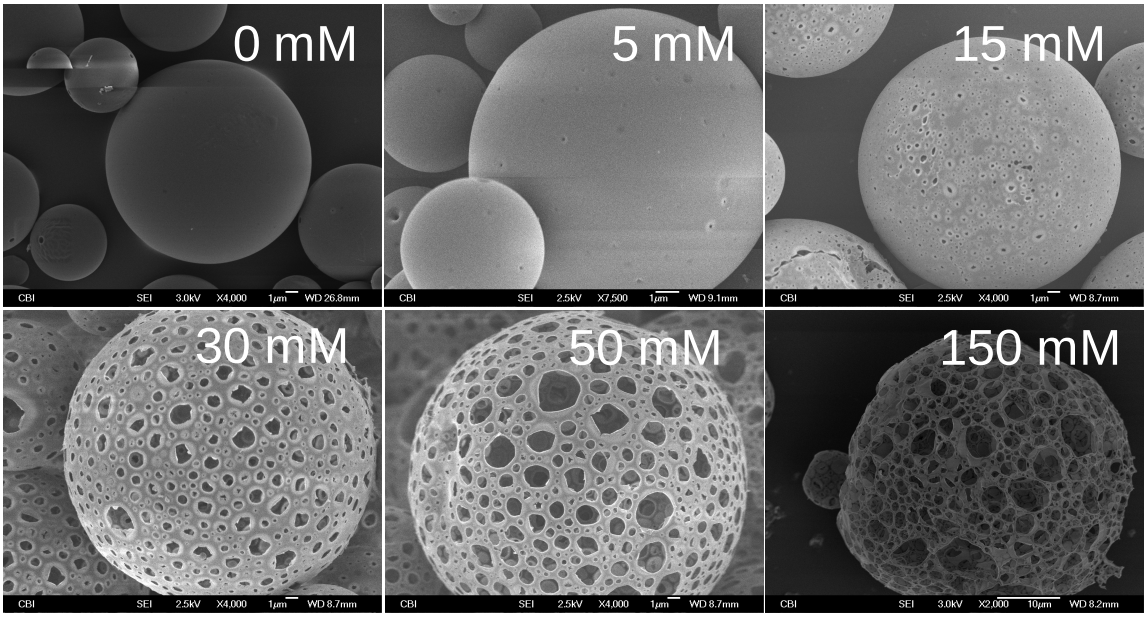

Figure 35: Scanning electron micrographs of microparticles prepared with varying amounts of NaCl in the primary emulsion.

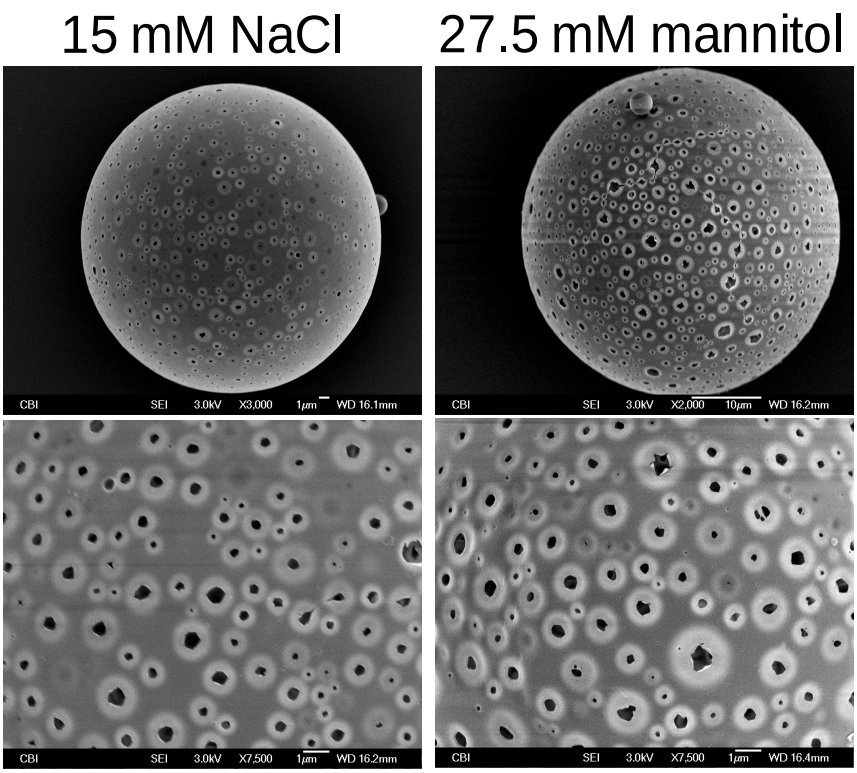

Figure 36: Scanning electron micrographs of microparticles prepared with equi-osmolal amounts of NaCl and mannitol.

Osmolarity measurements showed that 15mM NaCl and ~27.5 mM mannitol solutions have similar osmolarity (presumably because NaCl freely dissociates to Na+ and Cl- ions). Microparticles prepared with these concentrations of excipients have similar surface porosity as observed through these micrographs.

Interestingly, it was observed that particle porosity also depended on the polymer characteristics. Microparticles prepared under different osmotic gradients using the RG502H polymer (containing a free carboxy group at the polymer chain termini – acid number 6 mg KOH/g) were porous, while particles made with the RG502 polymer (containing an alkyl ester group at the polymer chain termini – acid number 1 mg KOH/g) were non-porous (Figure 37). Similar differences were observed when using PLA and PCL (more hydrophobic end groups

when compared to PLA) to fabricate microparticles (Figure 37). Such dramatic differences in the porosity of the particle surface could be due to differences in the hydrophilicity of polymer end-groups used. Presumably, microparticles with relatively hydrophilic surfaces (determined by the nature of polymer end-groups) allow for faster diffusion of water through the polymer to the inner emulsion, which leads to swelling and bursting of the inner emulsion and subsequent pore formation.

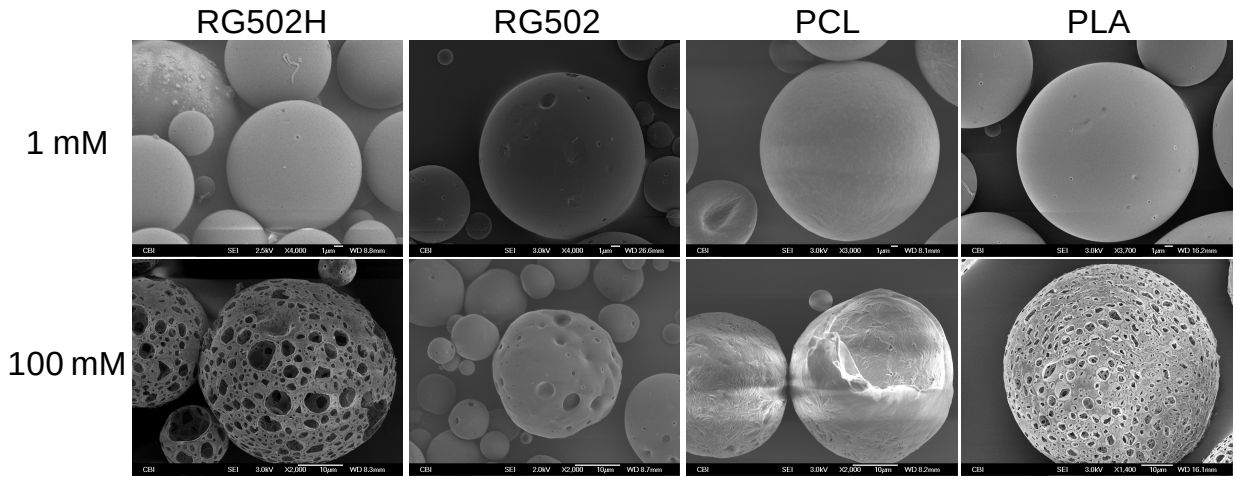

Figure 37: Scanning electron micrographs of microparticles prepared using different polymers.

Microparticle surface hydrophobicity (dictated by the nature of polymer end groups) may have a role to play in establishing particle porosity, as polymers with relatively hydrophilic end-groups (RG502H PLGA and PLA) form pores when osmotic gradients are created between the inner emulsion and outside, while polymers with relatively hydrophobic end-groups (RG502 PLGA, PCL) do not form pores.

Further, preliminary densities as well as surface area measurements were obtained from the porous particles to determine if any of them could potentially be used for pulmonary delivery of therapeutics (Table 2). As expected, we observe that the surface area increases while the density decreases, with increasing porosity of microparticles. The suggested density value

(which determines the aerodynamic diameter) required for deep lung deposition and pulmonary delivery of therapeutics is 0.4 g/cc [232]. This suggests that 50mM microparticles, which have a density of 0.25 g/cc, would be ideal for pulmonary delivery.

Table 2: Surface area and density measurements for porous microparticles prepared under different conditions.

All measurements are based on n = 1. Surface area was determined using a Quantachrome surface area analyzed, Avg. dia. using a Beckman Coulter Counter, and the Avg. density using an ultrapycometer

NaCl Conc. (mM)	Polymer	Surface Area (m^2/g)	Average Diameter (μm)	Average Density (g/cc)
1	502H	18.0	11.5 ± 4.8	ND
5	502H	69.8	11.5 ± 4.1	ND
15	502H	79.4	15.2 ± 7.4	ND
30	502H	109	16.5 ± 6.9	1.12
50	502H	246	18.8 ± 9.7	0.25
1	502	30.5	10.3 ± 3.8	ND
100	502	27.3	12.9 ± 4.6	ND

Finally, to determine if release kinetics can be altered by making microparticles porous, we encapsulated a chemokine, CCL22, in non-porous or porous particles. Release of the chemokine from these particle sets was measured over 4 weeks using a CCL22-specific ELISA. We observe that the porous particles release greater amounts of CCL22 continuously over the 4 week period (Figure 38), while the non-porous particles remain in the lag phase of release [159-160].

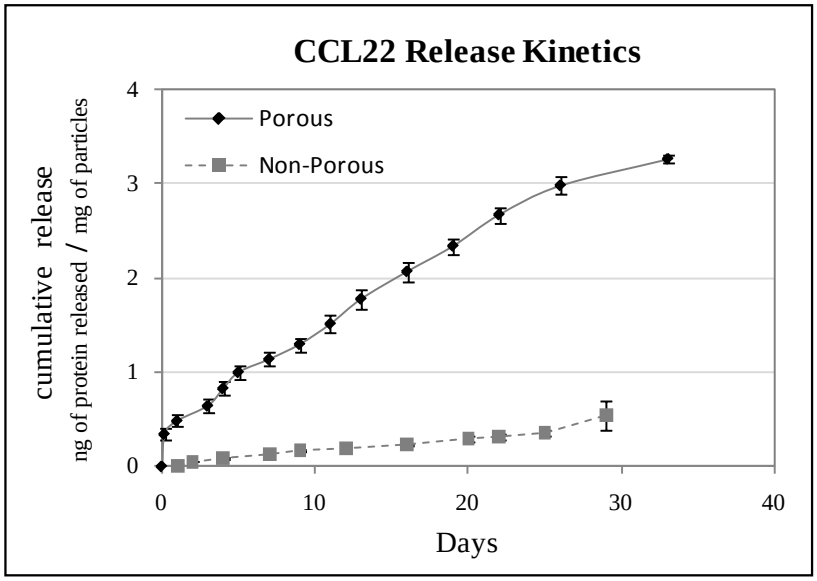

Figure 38: CCL22MP release kinetics.

Release kinetics of non-porous and porous CCL22 microparticles as measured in PBS. Porous CCL22MP release is the same as the one depicted in Figure 9.

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