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Micro and Nanoparticle Drug Delivery Systems for Preventing Allotransplant Rejection

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Abstract

Despite decades of advances in transplant immunology, tissue damage caused by acute allograft rejection remains the primary cause of morbidity and mortality in the transplant recipient. Moreover, the long-term sequelae of lifelong immunosuppression leaves patients at risk for developing a host of other deleterious conditions. Controlled drug delivery using micro- and nanoparticles (MNPs) is an effective way to deliver higher local doses of a given drug to specific tissues and cells while mitigating systemic effects. Herein, we review several descriptions of MNP immunotherapies aimed at prolonging allograft survival. We also discuss developments in the field of biomimetic drug delivery that use MNP constructs to induce and recruit our bodies' own suppressive immune cells. Finally, we comment on the regulatory pathway associated with these drug delivery systems. Collectively, it is our hope the studies described in this review will help to usher in a new era of immunotherapy in organ transplantation.

Keywords

Transplant Immunology; Microparticles; Nanoparticles; Biomaterials; Immunosuppression; Biomimetic; Controlled Release

1. Allotransplantation - From Mythology to Life Saving Medicine

The concept of replacing or transplanting damaged organs and tissues has likely existed as long as human disease and trauma [1, 2]. Initially only described as the medicine of

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mythology, it was not until the 1970s that effective combination immunosuppression was able to consistently yield successful long-term survival in early kidney transplant recipients. Further advances including the discovery of calcineurin inhibitor drugs (FK506 and Cyclosporine), the development of medical devices (such as the heart-lung machine), improvement in surgical techniques and a more mature understanding of the immune system has made the discipline of transplantation a ubiquitous and life saving therapeutic strategy [3].

The success of organ transplantation to date has hinged on the continued development (and improvement) of immunosuppression [3]. Though somewhat subjective, the development of immunosuppression (in the context of transplant immunology) can be divided into four distinct eras (Figure 1). The first era of immunosuppression employed rather rudimentary techniques including total body irradiation, splenectomies, thymectomies, and high dose corticosteroids [4]. The next era ushered in the development of more sophisticated pharmacological agents with more specific targets (Cyclosporine, FK506 and Mycophenolic Acid) [5–10]. Building upon these discoveries, research in the past twenty years focused the development of specific monoclonal antibodies and fusion proteins that target various lymphocyte ligands. The (future) fourth era of immunosuppression encompasses current experimental therapies being reported in small trials and preclinical studies in the literature. This includes the use of ex vivo cell therapy [11–14], organ engineering [15, 16], and smart immunosuppression using biomaterials such as microparticles and nanoparticles the later two will be the primary focus of this review.

2. Clinical Immunosuppression – Mechanism, Indications and Side Effects

Clinically, the process of allograft rejection is staved off by the prescription of immunosuppressive agents that are administered systemically. While effective at promoting allograft survival, the use of lifelong, high dose, multi-drug immunosuppression is associated with a well-established sequelae of global side effects [17, 18]. The following section will introduce some of the most commonly used immunosuppressants as well as their associated advantages and disadvantages.

2.1 Mycophenolic Acid (MMF)

Mycophenolic acid (MMF) is an anti-proliferative immunosuppression drug that is now generally used in most centers in lieu of the antimetabolite azathioprine due to its more favorable toxicity profile and specificity for lymphocytes. From a mechanistic standpoint, MMF is an inhibitor of inosine monophosphate dehydrogenase-2 (IMPDH2), which is the rate-limiting enzyme in *de novo* guanine synthesis. IMPDH2 activity is increased in highly proliferating cells as well as in tissues with rapidly proliferating cell populations (such as T cells and B cells). Complications with MMF include leukopenia, gastritis, esophagitis, and opportunistic CMV infection [19–21].

2.2 Calcineurin Inhibitors - FK506 and Cyclosporine

Cyclosporine (CsA), implemented by R.Y. Calne in 1979, dramatically changed the landscape of organ transplantation and is responsible for transplantation becoming the gold

standard treatment for end-stage organ failure. Mechanistically, CsA is able to suppress T cell activity via inhibition of the intracellular enzyme calcineurin, resulting in reduced expression of pro-inflammatory cytokines necessary for activity of effector T cells. Though efficient in reducing the incidence of acute rejection, CsA has several severe side effects including end-organ toxicity, diabetes, hypertension and neurotoxicity [8].

FK506 (also known as tacrolimus) was developed as a next generation calcineurin inhibitor in the advent of the success of CsA. Described in 1987 by Kino and colleagues, FK506 is a macrolide antibiotic produced by *Streptococcus tsukubaensis* [6]. Of importance, FK506 has been shown to be 10–200 times more potent than CsA, allowing for FK506 to be a superior alternative for preventing episodes of acute rejection, and even positioning FK506 to be used as monotherapy in kidney transplantation [22–24]. Similar to CsA, FK506 unfortunately has numerous side effects including opportunistic infection, diabetes, nephrotoxicity and neurotoxicity [17, 25, 26].

2.3 Rapamycin

Rapamycin (also referred to as sirolimus or rapa) was initially discovered in the 1970s as a fermentation product of *Streptomyces hygroscopicus* with antifungal properties [27]. It was not until the discovery of the calcineurin inhibitor FK506 that researchers began to reevaluate rapamycin as a potential immunosuppressive agent [28]. Mechanistically, rapa inhibits the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase. By inhibiting mTOR, rapamycin is able to regulate cell proliferation by preventing cell cycle progression from the G1 to the S phase and inhibit protein synthesis [28]. In addition to its ability to affect T cell proliferation, rapamycin is also able to influence dendritic cell (DC) function, driving DCs toward a more tolerogenic phenotype [29–31]. Furthermore, rapamycin has also been shown to synergize with cytokines such as TGF- β and IL-2 to expand naïve T cells toward a regulatory T cell (Treg) phenotype [32–36]. Because the anti-proliferative effects of rapa are not exclusive to cells of the immune system, it can interfere with fibroblast function resulting in impaired wound healing. Other adverse side effects observed with rapamycin include life threatening pneumonitis, proteinuria and in some instances diabetes [28, 37]. Despite these adverse effects, rapamycin has been shown to have negligible nephrotoxicity (a significant advantage over its calcineurin inhibitor counterparts) [28].

2.4 Limitations of Current Immunosuppressive Agents

While the current lineup of immunosuppressive agents that are administered to transplant patients allow for improved graft survival times, this is not without cost to the patient. As alluded to above, most forms of immunosuppression currently used are associated with significant toxicity and side effects including nephrotoxicity, opportunistic infections, diminished tumor immunosurveillance, serum sickness, anaphylaxis and neurotoxicity [17, 23, 25, 26, 28, 37]. Additionally, systemic delivery (either oral (P.O.) or intravenous (IV)) of these agents is often times associated with unpredictable blood levels of drug, leading to the presence of toxic (peak) or non-therapeutic (trough) concentrations. Taken together, organ transplantation is not technically a “curative” intervention, but rather one that forces patients

to trade one chronic condition (end stage organ failure) for another chronic condition (becoming a transplant recipient patient).

Another barrier to improved patient outcomes after transplantation is the issue of patient compliance and non-adherence to the recommended treatment regimens. This is not surprising based on the laundry list of side effects listed above as well as the daunting dosing frequency of conventional immunosuppression protocols. While, there are multiple variables that can impact the degree of non-adherence (socioeconomic status, gender, education level), the incidence of non-adherence in some studies is as high as 68% [38–40]. Moreover, patient non-adherence is associated with poor outcomes, graft failure and increased costs to hospitals, patients and payers [38].

Collectively, these shortcomings (namely toxicity and non-adherence), underscore the importance of improving current therapies and developing future technologies to improve patient and graft outcomes in the context of allotransplantation. Such technologies would ideally be able to target delivery of therapeutic agents to the tissues/organs of interest (obviating systemic toxicity issues) and provide sustained release of factors (eliminating the need for regular and frequent consumption). To that end, the remainder of this review will focus on targeted and/or sustained drug delivery using nano- and microparticles.

3. Nano and Microparticle Based Immunosuppression

The future success of the field of transplant immunology centers upon the resolution of many of the current shortcomings with conventional immunosuppression. Decades of research in the fields of materials science and biomaterials have resulted in the development of numerous drug delivery devices that aid current immunosuppressants in eliciting improved results via sustained, targeted and controlled release of drugs [41]. Specifically, controlled drug delivery offers several key advantages over systemic therapy such as the ability to maintain therapeutic drug concentrations, reduce systemic toxicity by targeting the therapy to specific sites (tissues or cells), and protect therapeutic payloads [42]. In addition, recent advances in the field have provided engineers with the necessary tools to create, biocompatible and biodegradable delivery systems that can target various tissue types and even triggerably degrade in response to specific environmental cues. The most widely used drug delivery systems are polymeric micro- and nanoparticles (MNP). Properties of MNPs such as size, and release kinetics can be tuned by adjusting the chemical composition, fabrication method, degree of crosslinking and molecular weight [43–45]. MNP size and chemical composition can also be modified to allow for depot release or intracellular uptake. Chemical modification of the surface of MNPs can allow for covalent coupling of targeting molecules such as antibodies and receptors. Taken together these features improve the clinical efficacy of pharmacological therapies by limiting systemic exposure to a given drug, while concurrently providing controlled, local delivery to the desired tissue (Figure 2A).

Mechanistically, the tissue and cellular distribution of the MNP systems described herein can be modified primarily via particle size. MNP size plays a critical role in the biodistribution of a given particle. Specifically, large microparticles (>10µm) can act as a depot at the site of injection as they are large enough to avoid phagocytic clearance and

cannot pass through most biological barriers [43]. Smaller microparticles (approximately 2–3 µm) are considered to be optimal for uptake by antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs), but large enough non-specific pinocytotic cellular uptake [46]. Given the versatility of these drug delivery systems, there has been much interest in optimizing and improving current immunotherapies (described above) by incorporating them in MNP systems. Though there are numerous studies detailing MNPs in cancer therapy, vaccine development and infectious disease [41, 43, 47], we will focus on the use of MNPs in transplant immunology (Table 1).

3.1 Calcineurin Inhibitor MNP Release Systems

As mentioned previously, CNIs currently remain the mainstay of most clinically employed immunosuppression protocols. Due to toxic side effects associated with systemic CNI therapy and poor oral bioavailability, there has been significant interest in developing CNI based MNP delivery systems [17, 48].

Sanchez *et al.* was among the first to develop, characterize and test CsA MNP formulations [49]. In these studies, three groups of poly(lactic-co-glycolic acid) (PLGA), MNPs (30 µm, 1 µm, and 0.2 µm) were fabricated. *In vivo* murine studies demonstrated that after subcutaneous injection, the 1 µm and 0.2 µm MNPs displayed a significant burst phenomena (i.e. high initial blood levels of CSA) whereas the 30 µm formulations lacked such a large burst, providing steady blood levels of drug for over three weeks [49–51]. Building on this study, Urata *et al.* developed control release CsA poly(L-lactic acid) (PLA) microparticles containing fatty esters capable of releasing CsA *in vivo* for up to 4 weeks (without an initial burst). These formulations were effective at reducing symptoms of arthritis when administered in a rat model [52].

Targeted delivery of CsA MNPs (to both cells and lymphatics) offers all of the advantages of having a depot release of CsA while simultaneously providing a higher local concentration of drug to the tissues and cells of interest. Yoshikawa *et al.* were able to design PLGA nanoparticles (average diameter 260 nm) that released CsA for 30 days [53]. Moreover, intramuscular injection of CsA nanoparticles (femoral region) yielded undetectable plasma levels of CsA, but concentrations in the femoral lymph nodes were nearly 20 times that obtained when administering soluble systemic CsA [53].

Previous studies have revealed that polyethylene glycol (PEG) coated PLA particles (when compared to non PEG coated PLA particles) administered orally delivered significantly higher amounts of radioactive antigen to the lymphatic's [54]. This effect was hypothesized to be a result of increased particle hydrophilicity after PEG coupling [45]. Building on this finding, Azzi *et al.* described a method for conjugating CsA to PLA/PEG nanoparticles (average size 84 nm). These CsA/PLA/PEG NPs were able to suppress T cell proliferation in mixed lymphocyte reaction (MLRs) in a comparable manner to soluble CsA. Moreover, they were able to show that DCs could effectively phagocytose and traffic NPs to the popliteal lymph nodes in a mouse model. Finally, NP-treated DCs were also able to suppress T cell proliferation (MLR) when compared to untreated DCs [55].

In addition to synthetic polymer-based MNPs, several groups have developed natural (biological material) based MNPs to release CsA. Natural materials can have advantages over their synthetic counterparts, including improved cytotoxicity, genotoxicity and hemocompatibility. Woo *et al.* described hyaluronic acid microparticles that exhibited superior dissolution, solubility and bioavailability characteristics when compared with soluble CsA [56]. Additional studies using chitosan and fibrin based nanoconstructs have been described, however applications to clinically relevant pathologies have not yet been explored [57, 58].

Controlled release formulations of FK506 have also been explored due to FK506's potency and superiority to CsA [23, 24]. Sustained delivery of FK506 from PLGA-based MNPs is reported to be effective at preventing liver and islet allograft rejection and mitigating the effects of colitis in rodent models [59–62]. Investigators have also developed FK506 releasing Poly-D,L-lactic acid (PDLLA) releasing microparticles that (when administered orally) home to the Peyer's patches and prolong small bowel transplant survival in a porcine model [63]. Significant research has also been conducted in the development of pH sensitive FK506 MNPs, which can deliver FK506 to the colon [64, 65]. These delivery systems have been used to treat murine models of inflammatory bowel disease (IBD); however, such technologies could be used in the context of small bowel transplantation [61, 62, 66].

Very recently Gajanayake *et al.* tested the ability of a triglycerol monostearate (TGMS) enzyme-responsive hydrogel loaded with FK506 to prevent hindlimb allograft transplant rejection in a rat model [67]. Specifically a one time injection of FK506 loaded TGFMS was shown to prolong hindlimb survival across a full MHC barrier for > 100 days. Furthermore, it was demonstrated that tissue levels of FK506 in treated animals were increased 10 fold over systemic levels [67]. While this study did not specifically utilize MNPs, it is possible to envision future applications of such a gel used in tandem (or as a carrier) with MNPs to provide dual delivery of immunosuppressive agents.

3.2 Rapamycin MNP Release Systems

Rapamycin based MNP systems are an especially attractive therapy as Rapa has been shown to not only inhibit T cell proliferation, but also inhibit DC maturation and function and enrich for suppressive FoxP3+ Regulatory T cells [28–31, 44]. As such, technologies taking advantage of both of these characteristics have been described in the literature. Forrest *et al.* described injectable rapamycin-loaded poly(ethylene glycol)-b-poly(epsilon-caprolactone) (PEG-PCL) micelle constructs that could provide over 6 days of *in vitro* release [68]. Due to the described effects that rapa can have on DCs, several studies have focused on targeted delivery of PLGA based MNPs to DCs [69]. Jhunjunwala *et al.* described rapa microparticles (3.4 µm) that are phagocytosed by DCs and release their payload intracellularly for up to three weeks. DCs treated with these rapa microparticles had a decreased ability to induce T cell proliferation, and showed down regulation of surface co-stimulatory molecules [69]. Two other studies examined delivery of rapa to DCs (both human and mouse) with smaller PLGA nanoparticles (150–450 nm). These studies demonstrated that rapa nanoparticles are effectively taken up by DCs and decreased expression of all maturation markers (Figure 2B). Furthermore, when compared with free

rapamycin, rapa nanoparticle-treated DCs showed decreased immunostimulatory capacity [70, 71].

In addition to PLGA based MNPs, there have been reports of alternative materials used in the development of rapamycin drug delivery systems. Kauffman and associates recently described rapamycin-loaded, acetylated dextran microparticles that have several advantages over PLGA microparticles such as more tunable release kinetics and *differential* release in acidic vs. neutral conditions [72]. Furthermore, Yuan *et al.* were able to develop rapamycin load chitosan/PLA nano particles to prevent rejection in a rabbit model of corneal transplantation. Chitosan/PLA rapa nanoparticles were able to show superior procorneal retention (as compared to free rapa) and were able to delay corneal allograft rejection [73].

3.3 MNP Delivery of Antimetabolites

In addition to intracellular delivery of rapamycin, there has been considerable interest in developing MNP based systems that can release the antimetabolite MMF. Shirali et al described an MMF PLGA based nanoparticle system that appears to prolong full thickness murine skin transplants across a full MHC mismatch (BALB/c → C57BL/6), when compared with daily systemic administration of MMF, via an apparent PD-L1 mediated mechanism. Notably, this therapeutic result was achieved despite MMF nanoparticles containing 1000 fold less MMF than is typically required with systemic administration of rapamycin. Moreover, treatment with MMF nanoparticles resulted in no detectable toxicity, whereas recipients receiving conventional MMF developed iatrogenic cytopenias [74]. In a similar study, MMF loaded in a nanoparticle containing cyclodextrins enclosed in a lipid bilayer (referred to as a nanogel), was capable of prolonging survival in a mouse model of lupus, when compared to free drug. The authors concluded that the enhanced ability of MMF nanogels to suppress inflammation could be attributed to nanoparticle uptake by DCs (Figure 2B) [75]. In yet another study from this group, researchers directly compared DC internalization ability of MMF PLGA nanoparticles to MMF nanogels [76]. The authors were able to show that while both MMF formulations were able reduce production of proinflammatory cytokines and stimulatory surface markers, the MMF nanogels were more effective, likely due to their enhanced uptake by DCs. As a caveat, while the MMF nanogels were more efficiently taken up by DCs, similar immunosuppressive effects could probably be seen with MMF PLGA nanoparticles at the expense of using a higher dose of nanoparticles [76].

3.4 MNP Delivery of Genetic Material for Immunomodulation

Given the boom of interest in of gene therapy, it should be of no surprise that this technology could be applied to immunomodulation and immunosuppression strategies. Specifically double stranded small interfering RNA (siRNA) or single stranded antisense oligonucleotides, can silence or down regulate expression of specific genes of interest in the inflammatory pathway. The fragile nature of these compounds in serum (due to the ubiquity of serum nucleases) makes direct infusion an inefficient delivery method [77, 78]. A potential solution to this problem would be to treat a patient's own cells *ex vivo* with a given type of gene therapy, followed by reinfusion of the treated cells. While obviating issues

concerning stability of said genetic material this approach is faced with the many cumbersome logistical and regulatory hurdles associated with cell therapy [79].

MNP systems represent effective carriers for genetic material as they can protect the payload and in some instances deliver the given material intracellularly allowing for efficient gene silencing. While gene therapy and delivery using MNPs has been described extensively in the literature for the treatment of a multitude of diseases, there are few studies examining the use of MNP-based gene delivery to suppress inflammation. Giannoukakis and Trucco have used DC's treated with anti-sense CD40, CD80, and CD86 oligonucleotides for treatment of type 1 diabetes; this cell therapy approach is currently in clinical trials [80]. Despite the success of this approach, Giannoukakis and Trucco have also described a microparticle based system for intracellular delivery of these same factors to DCs. Polyethylene glycol (PEG)/polyvinyl pyrrolidone (PVP) based microparticles loaded with the antisense oligonucleotides were prepared and injected subcutaneously to a site anatomically proximal to the pancreatic lymph nodes in non-obese diabetic mice [81]. These microparticles accumulated in both the pancreas and spleen after injection and could reverse type 1 diabetes in treated mice. Moreover, Tregs isolated from microparticle treated mice were able to prevent beta cell allorecognition when adoptively transferred to secondary recipients [81]. Given these convincing results, it is likely that this approach could be met with success if applied to organ transplantation as well.

4. Biomimetic Drug Delivery

Though controlled and targeted delivery of immunosuppressive drugs allows for more efficacious clinical outcomes with fewer side effects, advances in our understanding of the immunology of the immune system have allowed for the development of treatments that mimic the body's own tolerogenic mechanisms. Indeed it is known that the cells of our own immune system have evolved to utilize a whole host of strategies to naturally maintain immunological homeostasis and promote tolerance, representing a level of sophistication that dwarfs clinically utilized immunosuppressive treatments. Notably, regulatory T cells (Treg) and tolerogenic dendritic cells (tDC) are two hallmark examples of cells with such homeostatic capabilities [13, 82]. The local presence (or absence) of these cells has been linked to the absence (or presence, respectively) of destructive inflammation in a number of diseases [14, 83, 84]. Indeed most therapeutic strategies to harness these cells have thus far focused on *ex vivo* expansion followed by *in vivo* re-administration. Though promising in experimental models and small trials, clinical implementation of these approaches faces numerous logistical and regulatory hurdles. These include GMP isolation and handling of cells, achieving *ex vivo* proliferation of isolated cells, and maintaining stability of adoptively-transferred cells [82]. As such, there is significant interest in the biomaterials community to develop synthetic (MNP based) cell constructs to mimic the effects of Tregs and tDCs as well as enrich these naturally occurring cell populations at a given site in the body (Figure 2D) [44].

4.1 MNP Delivery of Treg-Inducing and Recruiting Factors

It is well understood that the phenotypic fate of naïve T cells is determined by both soluble paracrine signals as well as T cell/APC contact in the immune synapse. Due to the potent

immunoregulatory characteristics of Treg, strategies to induce naïve T cells to adopt a Treg phenotype have the potential to promote self tolerance in the context of autoimmunity, or acquired tolerance in the context of allotransplantation [33, 85–87]. MNP based systems that deliver cytokines and proregulatory factors to promote Treg induction and expansion have been described by several groups. For example, investigators have developed targeted anti-CD4 coated PLGA based nanoparticles that release leukemia inhibitory factor (LIF), an IL-6 like cytokine shown to induce FoxP3 expression in T cells [86, 88, 89]. These CD4 targeting LIF nanoparticles were capable of expanding FoxP3+ cells *in vivo* in treated mice and expanding nonhuman primate Tregs *in vitro* [88]. Additionally, these same nanoparticles prolonged vascularized heart allograft survival across a full MHC mismatch when compared with controls receiving no treatment[88]. In yet another study, investigators were able to prevent β -islets cell transplant rejection across a full MHC barrier and promote normoglycemia in diabetic mice by tethering LIF eluting PLGA nanoparticles to β -islets [90]. Jhunjhunwala et al. describe another mimetic MNP based system that can induce/expand Tregs. In this study, they demonstrated that the combination of TGF β , IL-2 and rapamycin, (both soluble and in sustained release PLGA microparticle formulations) could convert both mouse and human naïve CD4+ T cells to FoxP3+ Tregs *in vitro* [32]. Specifically, after 4 days of co-culture with naïve T cells, this triple cocktail microparticle therapy efficiently induced FoxP3 expression in 80% of T cells. Importantly these microparticle-induced Tregs were capable of robust proliferation and expressed key canonical Treg cell surface markers (CD25, FR4 and GITR) [32]. Based on the results to date, it is possible that such Treg expanding and inducing microparticle formulations could serve as an off the shelf therapy for suppressing aberrant inflammation in the context of transplant immunology and autoimmunity.

While the idea of regulatory cell expansion and induction is an effective strategy to promote homeostasis, recruiting endogenous regulatory cells with particle-based “homing” systems is another viable strategy to enrich cells in a given location[44]. Indeed this concept is exploited often in nature by cells that secrete chemokines (chemoattractant cytokines) to recruit cells expressing the corresponding chemokine receptors to the source of the gradient. As a hallmark example, it has been shown that a variety of tumor cells secrete the chemokine CCL22, which appears to effectively recruit Tregs, which express the associated CCR4 receptor [91]. This local recruitment of Treg to the site of the malignancy is thought to enable tumor-specific immune evasion. Based on such phenomena, it is possible to envision strategies to recruit Tregs using CCL22 and induce therapeutically-relevant, local immunological hyporesponsiveness [92]. For instance, pancreatic islet cells have been transfected with an adenovirus designed to over express CCL22, as a means to recruit Tregs to the pancreas and prevent autoimmune diabetes in a mouse model [93].

Building on these findings, controlled release of CCL22 from PLGA microparticles was shown to promote site-specific recruitment of endogenous Tregs *in vivo* (Figure 2D) [94]. Specifically, CCL22 microparticles were able to recruit adoptively transferred Tregs in a mouse model to the site of microparticle injection and concurrently delay rejection of transplanted allogeneic cells (which were also injected at the site of microparticle administration) [94]. Moreover, Treg homing to a locally placed depot of CCL22

microparticles has been shown to obviate the inflammatory effects of periodontal disease in both murine and canine models [95].

4.2 MNP Systems to Induce Tolerogenic DCs

Building on the concept of multifactor microparticle based delivery, Lewis et al. recently demonstrated that combined (intracellular and extracellular) delivery of cytokines and small molecules to DCs could prevent DC maturation, thereby inducing a more tolerogenic phenotype [96]. Two classes of PLGA microparticles were designed; phagocytosable microparticles loaded with either rapamycin or retinoic acid (RA) and unphagocytosable microparticles loaded with either TGF β or IL-10. Four distinct combinations of microparticles (Rapa/IL-10, Rapa/TGF β , RA/IL-10 and RA/TGF β) were tested, when cultured with DCs they were shown to decrease levels of surface expression of MHC II, CD80 and CD86 and were resistant to LPS stimulation. Furthermore, DCs treated with these dual microparticle systems were able to suppress T cell stimulation in mixed lymphocyte reactions and skew T cells toward a regulatory phenotype. Importantly, in all experiments it was shown that combinatorial pairings of immunosuppressive microparticles were more effective than microparticles releasing any of the respective single factors[96].

4.3 Artificial Antigen Presenting Cells

It is known that DCs play a key role in the initial steps of T cell activation, namely via antigen presentation (signal 1) in the context of signal 2 (costimulation) and 3 (paracrine cytokine signaling). Accordingly, there is great interest in developing synthetic particle-based materials to mimic the function of DCs in this process, allowing for specific T cell targeting for drug delivery [97, 98]. Referred to as “artificial antigen presenting cells” (aAPCs), these MNP particles seek to engage the TCR receptor in a similar manner as a natural APC and elicit a given response (Figure 2C) [99]. In general, most aAPC constructs have a high density of surface bound anti-CD3 and anti-CD28 ligands for the T cell receptor (CD3) and the costimulatory receptors (CD28) (signals 1 and 2). Further, there are descriptions of aAPCs that (in addition to the aforementioned surface coatings) are also loaded with cytokine (signal 3). Though anti-CD3 can serve as a nonspecific activator for all T cell receptors, there are also descriptions of aAPCs that can present a given peptide in the context of an MHC class II complex (analogous to what is observed in nature), thus providing an antigen specific response [100].

The ideal aAPC should possess three characteristics, namely it should be 1.) Easily modifiable to allow for a variety of surface modifications, 2.) Capable of controlled release of cytokines when engaged with a T cell and 3.) Available in an off the shelf formulation [99, 101]. Steenblock et al. were the first to described such an all-inclusive aAPC [99]. Using PLGA as the base polymer, these aAPCs consisted of surface bound anti-CD3, and anti-CD28 as well as encapsulated soluble IL-2 (signals 1, 2, and 3). Surface modification was carried out by incorporating an avidin-palmitic acid conjugate into the PLGA emulsion (thus allowing the palmitate to interact with the hydrophobic PLGA core and the avidin residing on the surface) allowing for biotinylated anti-CD3/CD28 or peptide/MHC complex conjugation to the particle surface. This combination of antigen presentation, costimulation and paracrine delivery of IL-2 was able to enhance *in vitro* CD8+ T cell proliferation by a

factor of 45 when compared with addition of exogenous IL-2 [99]. Building on this observation, further studies showed that the spatial and temporal characteristics of paracrine IL-2 release from an aAPC can have differential effects on CD8+ and CD4+ cells. Specifically, delivery of IL-2 upon aAPC contact with the TCR complex promotes IL-2 accumulation in the APC/T cell immune synapse, resulting in robust CD8+ proliferation, but activation induced apoptosis in CD4+ populations [101]. Interestingly, this result could not be replicated even with addition of 1000 fold exogenous IL-2, suggesting that a slow sustained release along with synaptic accumulation of IL-2 are responsible for this result [101].

Though the vast majority the applications of aAPCs in the literature focus on immunostimulation and effector T cell proliferation, it is easy to envision strategies that employ the use of aAPCs for immunoregulatory applications. Indeed, Francisco *et al.* were able to show that surface bound PD-L1 on Dynabeads along with soluble TGF β could enhance Treg induction when co-cultured with naïve T cells [102]. Schultz et al were the first to describe “killer aAPCs” that could target and delete antigen specific T cells [103, 104]. These killer aAPCs consisted of microparticles with the apoptosis inducing ligands (anti-Fas ligand (anti-FasL)) and HLA-A₂ affixed to the particle surface [104]. When co-cultured with T cell populations, these aAPCs were able to promote antigen specific T cell depletion in a FasL dependent mechanism [104]. In another study, “killer aAPCs” were shown prolong skin allograft survival (in a mouse model) via the deletion of antigen specific, alloreactive T cells [105]. Given these promising results, it will be interesting to see how these therapies translate to solid organ transplantation.

The interactions between DCs and T cells in the immune synapse is a complex process, and the results from the aforementioned studies (while promising) underscore this complexity and the necessity of continued understanding of these interactions. Indeed, paracrine factor accumulation between cells and nanoparticles as well as the thermodynamic interactions between MNPs and cells has been described in the literature [106, 107]. Studies of this nature will continue to provide the necessary clues to developing an optimal artificial cell construct. Given this complexity, the continued success in the development of effective biomimetic aAPCs for the next generation immunotherapeutics will rely on the continued collaboration between engineers and immunologists.

5. Translational Considerations

Though the previously-described technologies are only in the *in vitro* development stages or *in vivo* preclinical studies, it should be noted that there are numerous MNP-based drug delivery systems, for other applications, that are both FDA approved and in clinical trials [108]. These systems utilize many of the materials such as PLGA, PLA and PEG and lipid based liposomes. Nonetheless, these systems all consist of an existing FDA-approved drug packed into a drug delivery system [109]. The advantages of controlled and sustained release have been described (namely decreased toxicity and improved patient adherence), however translation of nonconventional drug delivery systems (including MNP loaded with genetic material, biomimetic protein delivery, and aAPCS) will likely be more cumbersome as these technologies would be treated as a new drug [110]. For example, translation of a

drug delivery system based on an existing FDA approved agent can cost between \$20–50 million dollars and take 3–4 years [110]. In contrast, development of a new drug can cost upwards of \$500 million dollars over 10 years [110]. Accordingly, it seems that in the immediate future, development of MNP systems that release FDA-approved immunosuppressive agents may have the easiest path to translation. This is not to say that the more complex delivery systems described are impractical, but a greater understanding of their toxicity profiles and clinical efficaciousness will need to be demonstrated before moving onto clinical trials.

While MNP systems that release FDA-approved immunosuppressives have an easier path to FDA approval, they also retain a host of issues that remain unresolved. The MNP systems currently approved by the FDA have a range of indications including delivery of chemotherapeutics, antipsychotics and antibiotics [109–111]. However there currently exist no MNP delivery system for immunosuppressive agents (for any application). The successful translation of such systems will hinge on demonstration of the safety of sustained release of highly toxic drugs such as FK506. Furthermore, delivery of immunosuppressive drugs using MNPs could present an issue if patient exposure to the delivered drug was to be discontinued (for example a transplant patient presenting with an active opportunistic infection). A potential solution to this problem would be to delivery MNPs in an injectable solution that could solidify after administration (such as a thermally responsive or shear responsive hydrogel)[112, 113]. The MNP gel complex would remain intact, provide sustained release of a given material and be easily removed in the event that therapy would need to be discontinued.

6. Concluding Remarks

The development of strategies for suppressing and modulating the immune responses following allotransplantation have improved considerably over the past 50 years. Rudimentary therapeutics such as corticosteroids and azathioprine that have global effects throughout the body have been replaced with more drugs that have T cell specific targets, with fewer (although still considerable) side effects. The development of both controlled, targeted delivery of these drugs as well as fabrication of biomimetic systems to modulate the immune system have the ability usher in a new era in transplantation in which allotransplantation may represent a true cure for end stage organ failure.

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Abbreviations

MNP	Micro- and Nanoparticles
MP	Microparticle
NP	Nanoparticle

aAPC Artificial Antigen Presenting Cell

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Highlights

- We describe developments in micro- and nanoparticle (MNP) based immunosuppression
- MNP drug delivery can provide sustained, controlled and local delivery to the site of an allograft
- Biomimetic MNP drug delivery can recruit, enrich and induce suppressor cells *in vivo*

Figure 1.
The progression of immunosuppression through four distinct eras.

Figure 2.
Delivery routes and mechanisms using MNP based drug delivery systems.

Table 1

Examples of currently developed MNP drug delivery systems for immunosuppression and regulation.

Small Molecule MNPs	Device Description	Key Findings	Ref.
	FK506 Eluting PLGA MPs	Subcutaneous injection of FK MPs could maintain therapeutic blood levels of drug and prolong murine liver and islet allograft survival.	58, 59
	FK506 Eluting PDLGA MPs	FK506 MPs accumulate in Peyer's Patches after oral administration and prolong swine small bowel transplant survival.	62
	FK506 Loaded TGMS Hydrogel	A single injection of enzyme responsive FK506 TGMS hydrogel prolongs rat hind-limb transplant survival when compared to systemic FK506.	66
	Rapa Loaded PLA/Chitosan NPs	PLA/Chitosan NPs release rapa for over 8 days. NPs demonstrated excellent retention in procorneal area and prolong corneal allografts (rabbit) when compared to rapa eye drops.	72
	Rapa Eluting PLGA MNPs	Rapa MNPs can be taken up by DCs for intracellular rapa delivery. Treated DCs showed decreased immunostimulatory capacity and decreased expression of maturation markers.	68–70
	MMF Loaded PLGA NPs	Intraperitoneal injections of MMF NPs prolong murine skin allografts (across a full MHC barrier) and show a decreased toxicity profile when compared to systemic soluble MMF.	73
	Antisense Oligonucleotide PEG/PVP MPs	Subcutaneous injection of MPs loaded with antisense CD40, CD80 and CD86 oligonucleotides reverse type 1 diabetes in NOD mice.	80
Biomimetic MNPs	LIF Eluting Anti-CD4 Coated PLGA NPs	LIF NPs coated with anti-CD4 target naive T cells, expand nonhuman primate Tregs (<i>in vitro</i>), expand murine Tregs <i>in vivo</i> and prolong murine heart allograft survival.	87
	CCL22 Releasing PLGA MPs	CCL22 MPs set up a chemokine gradient to recruit adoptively Tregs <i>in vivo</i> and can prevent inflammatory effects of periodontal disease in both murine and canine models.	92, 93
	Rapa, IL-2, and TGFβ PLGA MPs	The combination of RapamP/IL-2MP/TGFβ MP is able to induce naive T cells (mouse and human) toward FoxP3+ Treg phenotype <i>in vitro</i> .	32
aAPC MNPs	Anti-CD3 and Anti-CD28 Affixed to PLGA MP	IL-2 MPs with anti-CD3 and anti-CD28 coupled to the MP surface interact with CD8+ T cells <i>in vitro</i> and can induce robust proliferation.	97, 99
	Anti-Fas and MHC Antigen Affixed to Polystyrene MPs	Killer aAPCs coated with apoptosis inducing anti-Fas can selectively delete antigen specific T cells and prolong skin allograft survival in a mouse model.	102, 103