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Cellular and Molecular Targeting for Nanotherapeutics in Transplantation Tolerance

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Abstract

The induction of donor-specific tolerance to transplanted cells and organs, while preserving immune function as a whole, remains a highly sought after and elusive strategy for overcoming transplant rejection. Tolerance necessitates modulating a diverse array of cell types that recognize and respond to alloantigens, including antigen presenting cells and T lymphocytes.

Nanotherapeutic strategies that employ cellular and biomaterial engineering represent an emerging technology geared towards the goal of inducing transplant tolerance. Nanocarriers offer a platform for delivering antigens of interest to specific cell types in order to achieve tolerogenic antigen presentation. Furthermore, the technologies also provide an opportunity for local immunomodulation at the graft site. Nanocarriers delivering a combination of antigens and immunomodulating agents, such as rapamycin, provide a unique technology platform with the potential to enhance outcomes for the induction of transplant tolerance.

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Keywords

transplantation; tolerance; nanocarrier; targeting

1. Introduction

Cell and organ transplantation has become a standard procedure performed for the treatment of numerous end organ damage conditions, including cardiac, hepatic, and end-stage renal failure. Donor tissue is normally derived from an allogeneic source, which initiates an adverse host immune response by the recipient immune system. With the advent of modern immunosuppressive drug therapies, substantial improvements have been made in allograft survival in the past 20 years, though these enhanced clinical outcomes are typically due to short-term graft survival [1]. Chronic graft rejection and dysfunction persist despite long-term immunosuppressive drugs, with only 47-61% of grafts surviving to the 10-year mark [1, 2]. These life-long therapies are often harmful to the transplanted cells/organ and lead to non-specific suppression of the entire host immune system, resulting in patient susceptibility to infection and malignancies [3]. Further, patient quality of life is drastically impacted by long-term immunosuppression with side effects including headaches, gastrointestinal distress, hypertension, cataracts, hyperlipidemia, anemia, bone necrosis, renal damage, and arteriosclerosis [4].

The induction of antigen-specific tolerance to transplanted cells and organs to overcome immune-mediated rejection remains a primary objective. Tolerance implies that the host does not mount an immune response to the allogeneic graft, yet maintains full function for the remainder of the immune system. Tolerance necessitates modulating a diverse array of cell types that recognize and respond to alloantigens, including antigen presenting cells (APCs), T lymphocytes, and B lymphocytes. Targeting the alloreactive cells mediating rejection, as opposed to general immune suppression, is being enabled through advancements in nanotechnology. Nanotherapeutic approaches are being developed through cellular and biomaterials engineering for tissue and cell specific targeting within the body, delivery of immune-mediating factors (antibodies, cytokines, proteins), and synergy with current treatments. In this review we discuss the processes responsible for graft rejection and potential organ/cell targets for specifically modulating the immune response. We subsequently describe tolerogenic nanotherapeutic antigen carriers (Table I) and discuss potential design considerations, including target cell subsets and mechanisms associated with tolerance.

2. Factors mediating transplant rejection

The process of transplant rejection is initiated by recognition of donor antigens in the graft by the recipient immune system. The majority of these alloantigens belong to a class of proteins called the major histocompatibility complex (MHC), or MHC Class I antigens, which are found on the surface of all nucleated cells. MHC molecules present peptides on the surface of cells to T lymphocytes to generate an immune response, whether this is activation towards a pathogen or suppression to maintain peripheral tolerance. MHC antigens are highly polymorphic: within any individual, there are 100- to 1000-fold more

alloreactive T cells than T cells specific for other foreign antigens, creating a multitude of cell subsets contributing towards rejection [5, 6]. Even among optimally MHC matched donor-recipient pairs, transplant rejection often occurs due to minor histocompatibility (H) antigens, such as proteins encoded on the male Y chromosome not recognized by females [7, 8]. Hence, a multitude of donor antigens are responsible for transplant rejection, rendering tolerance induction historically difficult.

Recipient immune cells can respond to alloantigens presented by donor MHC molecules, termed *direct* allorecognition, or recipient MHC molecules, termed *indirect* allorecognition. Direct recognition typically occurs with the inadvertent and often unavoidable introduction of passenger leukocytes that accompany the cell or organ transplant, which prime T cells with direct donor specificity. This pathway plays a primary role in acute rejection immediately following transplantation. Conversely, alloantigens on transplanted tissues or organs can be processed and re-presented by recipient APCs in the context of recipient MHC molecules. Antigen from the graft is shed into the host environment for the lifetime of the graft, hence indirect allorecognition plays a dominant role in chronic rejection [9, 10]. Both the direct and indirect pathways of allorecognition contribute towards allograft rejection, though indirectly activated cells are involved in both acute and chronic rejection [11].

Both CD4⁺ T helper cells and CD8⁺ T effector cells mediate graft rejection, though their relative contributions among different transplant models are variable. CD4⁺ T and CD8⁺ T cells are capable of direct allorecognition of the donor cells/graft, leading to priming of other recipient immune cells and lysis of donor cells, respectively. Host CD4⁺ T cells are also involved in indirect allorecognition by cross-presenting donor antigen to CD8⁺ T cells, producing cytokines, and priming B cells for alloantibody production. Though CD4⁺ T cells are generally regarded as providing helper function to activate other components of the immune system, they can play an effector role as well in transplant rejection [12]. Because of this complex interplay and possible redundancy between CD4⁺ and CD8⁺ T cell subsets, an ideal tolerance approach should target both subsets of alloreactive T cells.

T cells can be primed for activation or inhibition by antigen presenting cells, such as macrophages or dendritic cells. T cells recognize an antigen-MHC complex presented by APCs, termed Signal 1, and their subsequent fate is determined by the presence/absence of a secondary costimulatory signal (Signal 2) and cytokine mediators (Signal 3) for differentiation into T helper cells, cytolytic T cells, T regulatory cells, *etc.* Positive costimulation promotes T cell activation and proliferation, such as CD28/(CD80 or CD86) or CD154/CD40 interaction between T cells/APCs. Costimulatory blockade has garnered success in rodent models in preventing transplant rejection, however, this effect has been difficult to replicate in large animals/non human primates [13]. Negative or inhibitory costimulatory pathways can be exploited to dampen immune responses, such as PD-1/(PD-L1 or PDL2) or CTLA-4/(CD80 or CD86) between activated lymphocytes/APCs. If T cells encounter presented antigen without Signal 2, this results in anergy or deletion, which are both desirable outcomes for the induction of tolerance.

3. Nano-scale antigen carriers

Nanotechnology and nanomaterials are a powerful technology for modulating cellular responses and have been widely used within the context of vaccines for immune activation. For this review, we consider nanocarriers as having diameters in the range of 1 to 1000 nm. Nanocarriers have been fabricated from a range of materials including polymers (*e.g.* PLGA, polystyrene), lipids (*e.g.* micelles, liposomes), gold, and carbon-based materials. These carriers can be loaded with antigen or drug by attachment to the surface (chemical conjugation or adsorption) or encapsulation within the carrier. Additionally, for promoting an immune response, these carriers typically have an adjuvant, such as aluminum salts or monophospholipid A (MPLA). Generating a specific, desired immune response using a nanocarrier is dependent on a number of factors, such as delivery route, size, shape/conformation, charge, incorporation of immune mediators, and presence/type of an adjuvant. For a detailed review on the above features, see McCarthy et al. [14]. Relative to traditional vaccines, nanocarriers offer the advantage of enhanced antigen stability, improved immunity, targeted delivery, and additional delivery routes.

More recently, antigen-loaded nanocarriers have been employed for tolerogenic approaches that dampen immune responses, with applications to autoimmune disease, allergy, and transplantation. Significant success was observed within the context of coupling antigens to cells as a delivery vehicle, and this strategy has more recently progressed to using polymer nanoparticles as carriers for the antigen. Initial advancements with tolerance induction have been within autoimmune disease models due to the presence of distinct, identifiable antigens, though these approaches have been extended to transplantation [15-17]. The following sections describe progress and opportunities regarding the induction of transplant tolerance with nanocarriers, which offer a platform for delivering antigens of interest, the capacity for specific cell targeting by incorporation of antibodies or ligands, and an opportunity for local immunomodulation through delivery of drugs or proteins.

4. Cellular and organ-specific targets for nanotherapies

Antigen presenting cells (APCs), which internalize and present alloantigens via the indirect pathway, are an attractive target for nanotherapies to generate a donor-specific tolerant state in immune cells. Nanocarriers containing antigens or immunomodulatory factors can be directed to specific receptors of tolerogenic APCs for uptake and re-presentation to CD4⁺ and CD8⁺ T lymphocytes. The APCs mediating alloantigen presentation include circulating or tissue-specific subsets of dendritic cells and macrophages. Of note, APCs within the liver, the spleen, and lymphoid tissue — liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), splenic marginal zone macrophages, lymphatic endothelial cells (LECs), and plasmacytoid DCs — have been documented in mediating tolerance [18-24].

4.1 Liver APCs

The liver represents a natural target for immune tolerance because i) the liver continually processes self and environmental antigens from dying cells and the intestine, respectively, and ii) antigen or nanoparticles delivered intravenously are naturally cleared by the liver. Many APCs within the liver are designed for immune tolerance rather than immune

activation, as the liver must continually clear naturally dying cells without mounting an immune response to self antigens. The liver contains conventional DCs as well as liver-specific APCs, including liver sinusoidal epithelial cells (LSECs), Kupffer cells (KCs), and hepatocytes. These cells are all able to present antigen in a tolerogenic manner, which is characterized by the production of anti-inflammatory cytokines and expression of negative costimulatory molecules [25].

KCs, the resident liver macrophages, are the main liver scavenger cells of endogenous cellular debris or nanoparticles. KCs produce IL-10 upon stimulation, express PD-L1, and cross-present foreign antigen in a tolerogenic manner [21, 26, 27]. Other liver APCs, such as hepatocytes and hepatic stellate cells, are capable of internalizing nanoparticles but less efficient at cross-presenting antigen than KCs [21]. Blockade of KCs has been shown to inhibit tolerance to rat cardiac allografts, and mechanistic studies indicate that KCs express lower levels of the costimulatory molecules B7-1, B7-2, and CD40 in comparison to DCs [28, 29].

LSECs represent a particularly attractive target for nanoparticles as well, as they are highly efficient at presenting foreign antigen to both CD4⁺ T cells and CD8⁺ T cells (via cross-presentation) [30, 31]. LSECs are highly efficient scavenging cells and are able to process apoptotic cell materials and soluble circulating Ag to CD8⁺ T cells, rendering them tolerant [31-34]. Importantly, LSECs have also shown the ability to tolerize alloreactive CD4⁺ and CD8⁺ T cells towards alloantigens *in vitro* and *in vivo* [35, 36]. Recently, LSECs have been targeted by a nanotherapy using a multifunctional envelop-type nano device (MEND) that successfully delivered siRNA for gene silencing [37]. Though this study aimed to suppress disease-related genes in LSECs, this approach shows that LSECs are an achievable target for targeted uptake of nanocarriers.

4.2 Splenic APCs

One of the primary functions of the spleen is to filter the blood and scavenge particulates without mounting an immune response, and splenic APCs have been implicated in the induction of tolerance in numerous models. The spleen is comprised of the red pulp, an area containing macrophages, DCs, and neutrophils, and a lymphoid white pulp containing T and B cells. The red pulp contains a highly phagocytic cell type called marginal zone macrophages (MZM) located near the circulation that express high levels of the scavenging receptors SR-A and MARCO (discussed in 5.2) [38]. MZMs are essential for tolerance to self, as reduced immune tolerance and promotion of inflammatory immunity occurs when MZMs are absent [38, 39].

Antigen covalently coupled to the surface of apoptotic splenocytes (Ag-SP) with subsequent delivery intravenously can induce tolerance to these antigens in murine models of multiple sclerosis, type 1 diabetes, allergy, and transplantation [40-43]. Tolerance is dependent on MZMs and IL-10 production, PD1/PD-L1 costimulation, and TGF- β -dependent regulatory T cells [40, 43]. MZMs and potentially other splenic APCs are critical for tolerance in the autoimmune R-EAE model, which is abrogated in splenectomized mice [40]. Additionally, in allogeneic islet transplantation, donor apoptotic cells have been shown to tolerize directly

activated T cells by anergy and indirectly activated T cells by clonal deletion and upregulation of negative costimulatory molecules on splenic DCs [15].

Dendritic cells also play a role in antigen internalization, presentation, and self tolerance within the spleen, notably CD8 α ^{neg}DCs in the red pulp and CD8 α ⁺DCs in the white pulp. CD8 α ^{neg}DCs are capable of high levels of MHC Class II expression and influence CD4⁺ T cells, while CD8 α ⁺DCs can cross-present antigen to CD8⁺ T cells [44, 45]. CD8 α ⁺ DCs have been implicated in tolerance initiation, and after administration of apoptotic splenocytes, CD8⁺ DCs phagocytose and suppress adaptive CD8⁺T cell inflammatory responses [46].

4.3 lymphoid and circulating APCs

Lymph nodes filter immune cells, Ags, and interstitial fluid from the periphery and are essential for both the activation of the immune response and maintenance of peripheral tolerance. Particle size has been identified as a critical factor in antigen uptake and retention in the lymph nodes, with nanoparticles of 25 nm or smaller more efficiently taken up and retained for longer periods than larger nanoparticles of 45 nm or greater [24, 47]. Lymphatic endothelial cells (LECs) are one of the first cell types in the periphery to contact foreign antigens, rendering them a potentially useful target in the context of transplantation antigens and allorecognition. Under homeostatic (non-inflammatory) conditions, LECs scavenge and cross-present foreign antigens to CD8⁺ T cells, resulting in upregulation of negative costimulatory PD-L1 and a tolerogenic phenotype [23].

Plasmacytoid DCs have also been targeted for tolerance, as nonstimulated pDCs play a role in regulatory responses [48]. Plasmacytoid DCs (pDCs) are found in the circulation and lymphoid tissues, typically contributing to inflammatory responses, DC maturation, Th1 activation, and CD8⁺ T cell activation [19, 49]. Plasmacytoid DCs have successfully been targeted by poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) to deliver encapsulated Ag, resulting in uptake and presentation of Ag to CD4⁺ and CD8⁺ T cells [19]. Ag-specific uptake *in vivo* by pDCs has suppressed CD4⁺ T cells and preserved regulatory T cells, suggesting a role for pDCs in tolerance [50].

5. Nanocarrier targeting for tolerogenic modulation in transplantation

Nanocarriers interact with APC subsets within the liver, spleen, and lymphoid tissue. Incorporation of ligands specific for receptors, including lectins and scavenging receptors, allows nanocarriers to target cell subsets. Targeting specific receptors has the potential to enhance internalization and Ag presentation, produce regulatory cytokines, and promote negative costimulatory pathways, which can generate tolerogenic responses.

5.1 C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) are expressed on DCs and macrophages and are responsible for Ag recognition, internalization, and re-presentation through recognition of specific carbohydrate structures [51]. One of the most commonly expressed lectin receptors on macrophages and DCs is the mannose receptor (MR or CD206), which binds to mannose found on self glycoproteins and pathogens [52]. Mannosylated nanoparticles and liposomes

have been extensively used to target the widely expressed MR, though in most cases for immune activation and vaccine applications [53, 54]. However, in the absence of an adjuvant, mannosylated liposomes cause increased uptake by DCs without upregulation of costimulatory molecules or T cell activation, skewing towards a tolerogenic phenotype [55]. Furthermore, administration of a soluble mannosylated myelin epitope reduced disease severity in an autoimmune mouse multiple sclerosis model, indicating that antigen delivery by MR can also induce suppressive immune responses [56].

Targeting DEC-205, another common CLR, may offer an advantage compared to MR regarding Ag uptake and presentation. Mahnke et al. showed that Ag complexed to anti-DEC-205 are presented 30-100 times more efficiently than anti-MR [57]. DEC-205 is expressed at high levels on DCs in lymphoid tissues, and antibodies toward DEC-205 have increased antigen uptake 400 times more than untargeted antigen [58, 59]. Several models have demonstrated that PLG nanoparticles (NPs) encapsulating Ag displaying antibodies against the CLRs DEC-205 or DC immunoreceptor (DCIR) were taken up by DCs compared to non-targeted NPs both *in vitro* and *in vivo* [19, 60]. Further, soluble proteins, when targeted to DEC-205 in the absence of an adjuvant, have led to deletion of antigen-specific T cells *in vivo* leading to tolerance [61, 62].

The C-type lectin Dectin-1, found on certain macrophages, neutrophils, and monocytes, illustrates the ability of receptor targeting to elicit distinct T cell responses [63, 64]. Carter et al. demonstrated that low doses of Ag delivered with anti-Dectin-1 Ab elicited a CD4⁺ T cell response, while Ag targeted with anti-DEC-205 favored a CD8⁺ T cell response [63]. Ligation of Dectin-1 has been shown to promote production of both the inhibitory cytokine IL-10 and a regulatory phenotype [65, 66]. Targeting different DC subsets via surface receptors can result in varied immune responses, offering the opportunity to tolerize the CD4⁺ and CD8⁺ directly and indirectly activated T cells mediating transplant rejection.

The CLR CLEC9A (C-type lectin domain family 9A, DNGR-1) recognizes and presents antigens from necrotic cells to CD4⁺ and CD8⁺ T cells, perhaps rendering it a useful receptor for tolerance. However, this receptor has also been targeted to generate anti-tumor responses [67, 68]. In humans, the CLEC9A receptor is found on DCs in the blood, spleen, and lymph nodes [69, 70]. Schreibelt et al. demonstrated that antigen-encapsulated PLG nanoparticles decorated with anti-CLEC9A antibodies were internalized by DCs with subsequent Ag cross-presentation. In comparison, control nanoparticles were internalized but did not cross-present Ag, indicating the CLEC9A-mediated uptake is required for Ag presentation by DCs [68]. Further, Ag delivered in the absence of an adjuvant via CLEC9A resulted in the differentiation of regulatory T cells and a tolerant phenotype [71]. Because CLEC9A is noted to present antigens from necrotic cells, this receptor is of particular relevance for the application of alloantigen presentation in the context of tolerance.

5.2 Scavenging receptors (SRs)

APCs commonly express scavenging receptors (SRs), a diverse group of receptors comprised of assorted proteins. SRs are known to bind and internalize bacterial pathogens, self proteins, and apoptotic cell debris. Receptors of interest involved in the routine

clearance of endogenous cell debris include the macrophage receptor with a collagenous structure (MARCO) and scavenger receptor class A (SRA) [72, 73].

MARCO enables binding and internalization of apoptotic cells by macrophages *in vitro* and has been implicated in tolerance induction through antigen-coupled cells and nanoparticles [40, 74, 75]. MARCO plays a key role in the clearance of apoptotic debris in the spleen, though the precise mechanisms are not understood [38]. Peptide-coupled polystyrene nanoparticles localize to the splenic marginal zone after intravenous injection and co-localize with MARCO⁺ cells [75]. MARCO is critical for tolerance induced by these nanoparticles in an autoimmune murine model of multiple sclerosis, as MARCO deficient mice were not capable of being tolerized [75]. The role of MARCO is further established by *Marco*^{-/-} mice, which have a reduced ability to clear bacteria and particles, increased inflammation, and upregulated cytokine release [76].

Another receptor involved with apoptotic cell clearance and a suppressive immune capacity is scavenging receptor A (SRA), or CD204, found on macrophages and DCs [77]. SRA binds to many ligands, both pathogenic and endogenous, and it has been reported to suppress antigen-specific T cell activation [78]. DCs in SRA deficient mice exposed to cell debris upregulate costimulatory molecules and inflammatory cytokines, suggesting a regulatory or suppressive role for SRA expressing APCs [78]. Interestingly, apoptotic cells are still cleared normally in SRA deficient mice, indicating a redundancy in receptors for apoptotic cell clearance [79]. This finding implies that nanoparticles can target one or more scavenging receptors for internalization by APCs involved in apoptotic cell clearance and tolerogenic responses.

The scavenging receptor DC-asialoglycoprotein receptor (DC-ASGPR), which is a lectin-like receptor, has recently been shown to generate antigen-specific suppressive CD4⁺T cells that secrete IL-10 *in vitro* and *in vivo* [80]. These suppressor cells were polarized from either naïve or memory CD4⁺ T cells upon delivery via DC-ASGPR of self or foreign antigens. Hence, targeting DC-ASGPR has implications for promoting regulatory activity in both transplantation and autoimmune disease.

6. Localized delivery of immunomodulatory agents by nanocarriers

Immunosuppressive drugs administered in the clinic for transplantation can induce regulatory function and the suppression of alloreactive cells, a desirable response for a tolerogenic therapy. However, many of these drugs, such as cyclosporin A and tacrolimus, non-specifically dampen immune responses to delay rejection, resulting in patient susceptibility to opportunistic infections and neoplasias. Nanocarriers offer a unique solution to locally deliver approved immunosuppressive drugs to specific cell types, invoking tolerogenic effects while avoiding high systemic concentrations and undesired, non-specific side effects.

6.1 Nanocarrier-mediated delivery of rapamycin

Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor also known as Sirolimus and Rapamune in the clinic, has been shown to suppress T cell activation, promote the

expansion of regulatory T cells, and induce regulatory T cells towards delaying transplant rejection [81, 82]. Rapamycin was first given FDA approval in 1999 for the prevention of acute rejection in kidney transplantation and has since been investigated for liver and lung transplantation, certain forms of cancer, and drug eluting stents [83-86]. Rapamycin's effectiveness is due to its documented inhibitory effects on T cells, B cells, and dendritic cells [87-89]. Systemic administration of rapamycin is hindered, however, because of its poor solubility in aqueous solutions and low bioavailability [90]. Incorporation into a nanocarrier avoids these concerns and offers a strategy to locally deliver rapamycin to alloreactive immune cells.

Rapamycin has been delivered from nanoparticles, impacting the function of APCs towards a tolerogenic function. PLG has been extensively used as an antigen or drug delivery vehicle, and rapamycin-loaded PLG nanoparticles have been synthesized and characterized with high loading efficiencies and release profiles over a period of several weeks [89, 91, 92]. Upon alloantigen stimulation *in vitro*, rapamycin-loaded PLG nanoparticles inhibited DC maturation markers, increased DC production of the immunosuppressive cytokine TGF- β , and inhibited subsequent proliferation of T cells [89]. In DCs generated *ex vivo* from human cord blood, rapamycin-loaded nanoparticles demonstrated downregulation of ICAM-1, a molecule expressed by mature DCs favoring a Th1 response, whereas blank PLG nanoparticles increased ICAM-1 expression [93]. Rapamycin, along with other regulatory cytokines such as IL-2 and TGF- β , has also been shown to favor the induction or expansion of Tregs [94]. A microparticle platform delivering IL-2, TGF- β , and rapamycin induced Tregs from murine and human naïve T cells *in vitro* with demonstrated suppressive function, which would be advantageous in a transplant environment [92]. A recent report by Maldonado, et al. described the induction of tolerance using biodegradable nanoparticles carrying either protein or peptide Ags and rapamycin [95].

Other nanocarriers have demonstrated efficacy in delivering bioactive rapamycin [96-98], such as orally administered rapamycin nanoparticles that decreased tumor growth in pancreatic cancer xenografts [96]. Micelles encapsulating rapamycin have demonstrated antitumor activity in both an *in vitro* and *in vivo* model of colon cancer, and rapamycin nanoparticles have inhibited tumor growth in human lung cancer in mice [98, 99]. Application of a nanoscale rapamycin delivery towards transplantation could generate suppressive immune responses, specifically towards alloreactive CD4⁺ and CD8⁺ T cells mediating transplant rejection.

6.2 Nanocarrier-mediated delivery of tacrolimus

Tacrolimus is another immunosuppressant used in transplantation that has been investigated for suppressive purposes via nanocarrier delivery. Tacrolimus, a calcineurin inhibitor, is used in a variety of transplantation models, including liver, kidney, heart, pancreas, and bone marrow transplantation, and it functions by blocking IL-2 production and subsequent T cell proliferation [100-102]. PLG has been used to incorporate tacrolimus with up to 60% encapsulation efficiencies and successful targeting to the lymph nodes [100]. Tacrolimus MPEG-PLA nanoparticles prolonged rat liver allografts compared to administration of tacrolimus capsules and achieved an increased retention time in blood plasma [103].

Micelles have also been used to deliver tacrolimus, though they were only able to prolong tail skin allograft survival when in combination with rapamycin micelles whereas micelles containing tacrolimus alone had no effect on graft survival [97].

6.3 Synergistic nanotherapeutic approaches for transplantation

In the clinic, transplant recipients often receive a combination of immunosuppressive drugs, steroids, and other therapies that aim to 1) synergize with one another to enhance immunosuppression and graft survival, 2) enable reduced dosing/time course of drugs, and 3) minimize nephrotoxicity and other side effects [104]. Rapamycin has been investigated for synergy in immune suppression and tolerance induction in several models when combined with a second immune-mediating agent, yielding effects not possible with each therapy separately.

Rapamycin's synergistic effect has been documented with tacrolimus, doxorubicin, cyclophosphamide, and cyclosporin A in models of allogeneic skin, small bowel, bone marrow, and islet transplantation [17, 92, 97, 105, 106]. Definitive evidence of synergy between immunosuppressive drugs in transplantation has been shown with rapamycin and cyclophosphamide, a chemotherapy drug, in bone marrow transplantation. Mice receiving a bone marrow transplant and rapamycin or cyclophosphamide alone rejected the bone marrow graft, whereas a combination therapy of both drugs resulted in transplant tolerance as evidenced by stable donor cell engraftment [106]. A similar result is seen with rapamycin and cyclosporin, where subtherapeutic doses of the two drugs allow for extended allograft survival in rat heart and kidney transplantation compared to each alone or an additive effect [107].

Nanocarrier-mediated delivery of factors can enhance this synergy through cellular targeting. Rapamycin and doxorubicin delivered from nanocarriers has shown a unique synergism in a triple-negative breast cancer model (TNBC) [108]. Rapamycin-loaded micelles and liposomes incorporating doxorubicin, a chemotherapeutic drug, demonstrated a synergistic effect in both *in vitro* cytotoxicity and *in vivo* tumor inhibition. A combination index was calculated to quantitatively establish a synergistic, rather than additive, effect of this combined drug delivery system in tumor inhibition [108]. Towards targeting alloreactive cells, treatment by micelles containing rapamycin and tacrolimus prolonged survival of MHC-mismatched tail skin grafts from 16 days to 30 days [97]. Importantly, micelles containing tacrolimus alone did not extend graft survival, and only rapamycin alone exhibited a more modest extension of graft survival, from 16 days to 23 days. Hence, the combinatorial or synergistic effect was present only with the delivery of both rapamycin and tacrolimus [97].

In addition to combining immunosuppressants within nanocarriers, the synergism of immunosuppression with an antigen-specific approach has proven effective in transplantation tolerance. In an allogeneic model of islet transplantation, nanoparticles delivering donor antigens alone were able to induce tolerance in ~20% of recipients, and low dose systemic rapamycin alone at the time of transplant resulted in ~10% tolerized recipients [17]. However, the combined approach of nanoparticles delivering antigen with low dose rapamycin resulted in ~60% of recipients with long-term tolerance [17]. Thus, nanocarriers

can be used to deliver a combination of immunosuppressive drugs, proteins, or antigens that create synergistic effects towards the goal of inducing transplant tolerance.

7 Concluding Remarks

The induction of donor-specific transplant tolerance will enable a recipient to tolerate the allogeneic graft, or non-self, while maintaining full functioning of the remainder of the immune system. Due to the complexity and redundancies in transplant rejection, the mechanisms governing tolerance are incompletely defined. The use of nanotherapeutic approaches can enable a greater understanding of peripheral tolerance mechanisms occurring at an organ level — within the spleen, liver, and the graft — and cellular level as well as the relative contributions of cell subsets towards tolerance and their temporal importance respective to the time of transplant. Nanocarriers can target APCs and other cell subsets mediating rejection via cellular receptors while simultaneously delivering synergistic factors and/or antigens to generate tolerogenic and/or immunosuppressive responses. Several nanocarriers have shown efficacy *in vivo* with receptor targeting or delivery of factors, however, establishing an effective nanotherapy for transplant tolerance will likely require a strategic combination of these features. The field of nanotherapeutics represents a unique opportunity for immunomodulation and the induction of transplant tolerance.

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Abbreviations

Ag	antigen
LSEC	liver sinusoidal endothelial cell
LEC	lymphatic endothelial cell
CLR	C type lectin receptor
SR	Scavenger receptor

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Highlights

- We discuss the processes responsible for graft rejection and potential organ/cell targets for specifically modulating the immune response.
- We describe tolerogenic nanotherapeutic antigen carriers
- We discuss potential design considerations, including target cell subsets and mechanisms associated with tolerance.

Table I**Nanocarrier Approaches to the Regulation of Transplant Tolerance**

Nanocarrier Intervention	Receptor target	Anatomic Location	Model	Effect	Ref.
Mannosylated liposomes	Mannose Receptor (MR/CD206)	Macrophages, DCs	<i>In vitro</i>	↑ Human DC uptake; No T cell activation	White et al 2006
PLGA nanoparticles	DEC-205 (CD205)	DCs in lymphoid tissue, pDCs	<i>In vitro</i>	↑ DC uptake; Cross presentation to CD4 ⁺ and CD8 ⁺ T cells	Tel et al, 2013 Cruz et al, 2014
PLGA nanoparticles	CLEC9A/DNGR-1	DCs in blood, spleen, lymph node	<i>In vitro</i>	↑DC uptake; Cross presentation to CD4 ⁺ and CD8 ⁺ T cells	Schreibelt et al, 2012
PLGA nanoparticles encapsulating rapamycin	Undefined	DCs	<i>In vitro</i>	↓Maturation markers - MHC II, CD86, CD40; ↑TGF-β secretion; ↓T cell proliferation	Haddadi et al, 2007
Polystyrene, PLGA nanoparticles	MARCO	Splenic marginal zone macrophages	<i>In vivo</i>	Tolerance in R-EAE; Ag-specific T cell anergy, ↑ Tregs	Getts et al, 2012
PLGA nanoparticles	Undefined	DCs in liver and spleen	<i>In vivo</i>	Tolerance to mouse islet allografts; Clonal contraction of indirectly activated T cells	Bryant et al, 2014
PLGA nanoparticles encapsulating rapamycin	Undefined	DCs in liver and spleen	<i>In vivo</i>	Inhibition of CD4 ⁺ and CD8 ⁺ T cells and B cells; Tolerance in EAE and hemophilia A mice	Maldonado et al, 2014
MPEG-PLA nanoparticles encapsulating tacrolimus	Undefined	Undefined	<i>In vivo</i>	Prolonged survival of rat liver allografts	Xu et al, 2014
Micelles encapsulating tacrolimus + micelles containing rapamycin	Undefined	Undefined	<i>In vivo</i>	Prolonged survival of mouse tail skin allografts	Dane et al, 2011