Human Vaccines 7:1, 37-44; January 2011; © 2011 Landes Bioscience

Multifunctional dendritic cell-targeting polymeric microparticles

Engineering new vaccines for type 1 diabetes

Benjamin G. Keselowsky,[1,*] Chang Qing Xia[2] and Michael Clare-Salzler[2,*]

[1]J. Crayton Pruitt Family Department of Biomedical Engineering; [2]Department of Pathology; Immunology and Laboratory Medicine; College of Medicine; University of Florida; Gainesville, FL USA

Key words: vaccine, diabetes, dendritic cell, microparticle, microsphere, immunotherapy

Type 1 diabetes (T1D) is an autoimmune disease characterized by T-cell mediated destruction of insulin-producing β-cells.[1] Approximately 13,000 new cases of T1D are diagnosed each year in US children, with a prevalence of about 2 cases per 1,000 individuals, and associated treatment and care costs are large.[2] Following clinical diagnosis, many T1D patients maintain functional pancreatic β-cell mass for some time.[3] This therapeutic window has motivated attempts to halt autoimmune inflammation of pancreatic islets before too many islets are destroyed to maintain glucose regulation. Schemes involving broad immunosuppression[4] or antigen non-specific immunomodulation[5] have been explored for T1D treatment, however, only antigen-specific approaches hold the unique promise of long-lasting disease remission in the absence of adverse side effects.[6] Therefore, efforts have focused on manipulating the body's most efficient antigen-presenting cell, dendritic cells (DCs), in T1D and other autoimmune diseases.[7] Early and ongoing efforts involving DCs therapeutically focus on the exogenous generation of DCs for administration as a cellular vaccine.[7,8] In fact, an exogenously manipulated DC-based vaccine is currently being investigated in clinical trials for application in T1D.[9] However, it is generally accepted that cell-based vaccines for T1D are primarily intended to provide proof-of-concept, as several factors limit this approach.[10] In particular, dissemination and lymph node homing of exogenously delivered DCs is inefficient[11] and treatment involves isolation and storage of DCs under stringent manufacturing standards, amounting to high costs which prohibit widespread application.[12,13]

An attractive alternative strategy involves the in vivo targeting of DCs with injectable polymeric, biodegradable microparticles delivering a payload of vaccine components and immunomodulatory factors. Microparticle systems may be easily administered in a single injection to patients with extended delivery of both prime & boost doses using timed-release materials.[14,15] Furthermore, polymeric microparticle strategies greatly simplifies issues related to manufacturing, storage and shipping, as microparticle encapsulation affords stability for off-the-shelf availability.[16,17] Microparticles can be engineered to be multifunctional and modular, where features of particular interest are: (i) targeting to DCs, (ii) providing a depot for antigen, (iii) providing controlled delivery and subcellular targeting of adjuvants, immunosuppressants, chemokines or other conditioning factors. Conceptually, microparticle systems can thus be designed to attract DCs and precursors into an injection site, provide signals to promote differentiation into DCs, promote uptake of antigen and provided immunosuppressive/tolerogenic conditioning of DCs to induce specific tolerance. In practice, the amalgamation of these design principles are just beginning to be assembled for application toward T1D. The goal of this review is in part to showcase recent microparticle-based efforts toward immunosuppression and T1D mitigation in particular, but also to draw on the broader literature of DC-targeting of microparticles, largely applied to infection or cancer, as instructive examples to highlight the repertoire of tools available for the scientist/clinician investigating T1D therapies.

*Correspondence to: Michael Clare-Salzler and Benjamin Keselowsky; Email: salzler@pathology.ufl.edu and bgk@ufl.edu
Submitted: 06/25/10; Accepted: 07/05/10
DOI: 10.4161/hv.7.1.12916

Tolerogenic Dendritic Cells

The rationale for therapeutic interest in DCs is founded in their physiological roles. Dendritic cells are phagocytes and the most efficient antigen presenting cell.[18] Moreover, DCs are central regulators of the immune system, processing and presenting antigen to direct induction and expansion of specific T-cell subsets (e.g., Th1, Th2, Th17, regulatory T-cells), promoting either antigen-specific tolerance or immunity.[19,20] Key characteristics of DCs include: (i) their ability to uptake and transport antigen from peripheral tissues to T-cell zones in secondary lymphoid organs, (ii) activation marked by upregulation of major histocompatibility complex II (MHC II), co-stimulatory surface molecules (e.g., CD80, CD83, CD86), (iii) Ag processing and presentation on both MHC II and MHC I complexes and (iv) T-cell interaction and stimulation.[18,20] Furthermore, DCs play a critical role in both the establishment of central tolerance and the maintenance of peripheral tolerance. Mechanisms by which DCs facilitate immune protection in homeostasis include induction of T-cell deletion, anergy or regulatory T-cells (Tregs) for peripheral tolerance.[7,21-24] General (but not requisite) characteristics of tolerance-promoting/tolerogenic DCs that have been

reported include low expression of stimulatory and co-stimulatory molecules, low production of inflammatory cytokines (e.g., IL-12), and increased production of immunosuppressive cytokines (e.g., IL-10); with various reports of specific markers such as high levels of inhibitory molecules (e.g., programmed death ligand, immunoglobulin-like transcript-3) and indoleamine 2,3-dioxygenase (IDO).[21,25-29] There is enormous potential use of tolerogenic DCs as therapeutic agents to mitigate autoimmune diseases and transplant rejection.[7,25] Of particular interest for durable antigen-specific tolerance are tolerogenic DCs which can induce $CD4^+CD25^+Foxp3^+$ Tregs. These regulatory T-cells have the ability to suppress the actions of effector T-cells and also impair the activation of DCs.[23,24] A number of biological and pharmacological agents have been shown to induce DCs capable of generating $CD4^+CD25^+Foxp3^+$ Tregs and halt or reverse autoimmunity in animal models.[7,22] Conceivably, any of these factors or combination of factors are readily incorporated into microparticle formulations, some of which have already been reported, as discussed below.

Polymeric Microparticles for Targeted Antigen Delivery

Numerous polymeric biomaterials have been investigated in particle-based drug delivery vehicles to deliver immunomodulatory, functionally-active proteins, peptides, nucleic acids, antigens and adjuvants.[30-36] Of these, microparticles fabricated using biodegradable poly(lactide-glycolide) (PLGA) have been the most investigated vehicle for delivering immunotherapeutics and the influence of PLGA microparticles on DCs has been characterized.[14,37-43] Many biomaterial options exist for particle formulation, with various advantages.[44] However, PLGA, is appealing in terms of translation, as it has been approved by the US Food and Drug Administration for numerous devices including resorbable surgical sutures and drug delivery products, and is degraded in the body into natural products of lactic and glycolic acid. By altering the composition, polymeric microparticles can be designed to provide a tailored initial burst of the encapsulated immunomodulatory molecule followed by sustained release, permitting the design of a one-time drug administration with prime and boost doses.[14]

The most common technique to fabricate polymeric microparticles encapsulating molecules is the double-emulsion solvent-evaporation method.[14,30,45] Using this technique, a hydrophilic molecule (e.g., protein, peptide, nucleic acid) to be encapsulated is placed in aqueous solution while the polymer and a hydrophobic molecule (e.g., immunosuppressive drugs) to be incorporated is dissolved in an organic solution. The two phases are emulsified by sonication or homogenization and this primary emulsion is then added to a second larger, bulk aqueous phase and again mixed by homogenization to form a water-in-oil-in-water double emulsion. The organic solvent is then evaporated and the polymer-containing droplets harden to form microparticles, which are then isolated by filtration or centrifugation. Finally, the particles are lyophilized to remove water from the interior aqueous phase to result in a dry suspension of the encapsulated material within the polymer matrix. Additionally, microparticles can be surface-modified. For example, surface immobilization of a polycationic polymer allows surface-loading of nucleic acids or cell surface receptor-targeting molecules such as antibodies can be immobilized to promote DC interactions.

Microparticles of appropriate size (generally 1–5 μm) are too large to be taken up by cells through pinocytosis/endocytosis (by which nanoparticles are taken up), but are phagocytosed efficiently by specialized phagocytes.[30,46] This size exclusion of particulate uptake by cells provides a simple, but powerful passive targeting mechanism to deliver a payload of antigen and immunomodulatory factors to phagocytic cells such as DCs.[47-49] Protein-based antigen choices for microparticle formulations include whole protein antigens (e.g., insulin[50-52]) or specific antigenic peptides.[53-55] An advantage to whole protein antigens is it is not necessary to determine the immunodominant peptides, which may vary from patient to patient, while use of defined antigenic peptides carries less risk of an adverse immune response. Protein degradation within DC endosomes involves acidic hydrolysis, reactive oxygen species and enzymatic degradation, the mechanisms and consequences of the timing of which are being uncovered.[56] Critically, encapsulation of antigen in microparticles (e.g., PLGA) provides the advantages of enhancing and prolonging antigen presentation on both MHC-II and MHC-I molecules, requiring orders of magnitude less antigen compared to a soluble dose and providing antigen presentation for days-to-weeks, compared to hours-to-days for soluble antigen.[47,48,57,58] Loading of microparticle encapsulated antigen onto MHC-II complexes occurs in phagosomes.[59] On the other hand, loading onto MHC-I molecules through cross-presentation of exogenously-delivered antigen occurs via endosomal escape into the cytosol.[57,58] Microparticles of PLGA have the demonstrated ability to provide effective cross-presentation, shown to require active proteasome, indicating access to the cytosol,[60] possibly because polymer degradation acidifies the endosome, increasing the osmotic pressure to the point of leakage,[58,61] or through an alternate MHC-I processing pathway.[62,63] The molecular weight of the PLGA used to form microparticles can direct the kinetics of delivery to the endosome and subsequent release to the cytoplasm, with the lower molecular weight polymer (6 kDa) delivering an encapsulate after 24 h, while endosomal release generated from the higher molecular weight polymer (60 kDa) was delayed to day 5.[64]

Microparticle-associated endosomal escape has also been capitalized upon to effect non-viral delivery of nucleic acids to the cytosol for subsequent nuclear localization.[30] Microparticle incorporation of nucleic acids such as DNA plasmids, antisense oligodeoxynucleotides and small interfering RNA has been well-established, and provides protection from degradation by nucleases, as well as effective nucleotide delivery for gene expression or knockdown.[65-67] Gene delivery for expression of antigenic proteins is also an established means of establishing an antigen depot, generally referred to as DNA vaccines.[68] As applied to autoimmunity, DNA-based vaccines with genes encoding for autoantigen

have been referred to as an "inverse vaccination", and are being investigated clinically for multiple sclerosis and T1D.[69] In cases where endogenous antigen presentation by DCs is considered favorable (i.e., MHC-I loading for presentation to $CD8^+$ T-cells), DNA-based vaccines are expected to benefit from incorporation into microparticles for targeting and delivery. A number of polymeric microparticle systems have been developed to deliver DNA to cells, primarily incorporating polycationic molecules to facilitate DNA loading and nuclear translocation.[70,71]

Taken together, polymeric microparticles have demonstrated versatility to provide an antigen depot for vaccines and biomaterial properties are easily tailored to suit the design constraints set by the form of the antigen. An antigen depot can be passively targeted to DCs through microparticle size, and subcellular sites can be further targeted for payload delivery to the phagosome and cytosol as well as translocation into the nucleus. However, issues remain related to reduced protein stability when encapsulated in acidic microparticles such as PLGA. Denaturation of protein antigen is expected to reduce conformationally-dependent antibody responses, whereas, T-cell mediated responses, relying on recognition of processed antigen, are not as sensitive to a harsh carrier environment.[72,73] In order to improve encapsulated protein stability, the introduction of protein stabilizers has been explored.[74]

Tuning Microparticle Properties

Controlling the loading and degradation rate of polymeric systems is an important parameter that can affect immune responses. For instance, the level of antigen loading into polymeric microparticles is important as antigen dosing at both the high (e.g., in autoimmune encephalomyelitis[75]) and low extremes have been shown to either maintain ignorance or to induce tolerance.[76-79] Tuning the rate of polymer degradation in antigen-loaded microparticles is an important parameter, optimization of which currently requires empirical determination for a given application. For example, in the case of antigenic peptide loading into PLGA microparticles for rabies vaccination, the faster-releasing formulation was found to provide protection superior to complete Freund's adjuvant.[80] In contrast, slower-degrading ovalbumin-loaded PLGA microparticles induced longer-lasting anti-ovalbumin antibody titers.[81] On the other hand, low levels of prolonged antigen delivery are capable of inducing an "exhausted" T-cell phenotype, and in some (location-dependent) cases, tolerance.[82,83] It should be noted, however, that composition of the PLGA copolymer in itself could potentially affect DC response, as PLGA microparticles with a 50:50 ratio of lactide to glycolide been reported to maintain DC immaturity,[43] while a 75:25 ratio has been reported to induce DC activation.[41] It has been postulated that this effect may be explained by the fact that the higher lactide-containing polymers possess an increased surface hydrophobicity, which may conceivably present hydrophobic moieties interacting with receptors involved in danger signal recognition.[84] However, this issue remains to be resolved, as conflicting evidence regarding this trend has been reported.[85]

Because PLGA degrades relatively slowly, even at lysosomal pH, it has been posited that newer materials for microparticle vaccine systems which have been designed to primarily release their payload only when in phagosolysosomes, will prove advantageous.[44] For example, DC-targeting microparticles fabricated with materials incorporating pH-responsive elements such as cleavable acetal linkages[86,87] and orthoesters[88] have been investigated. These materials capitalize on the fact that extracellular pH is an average of 7.4, while phagosolysomes reach ~pH 5,[89,90] and have been used to provide high levels of DC-mediated $CD8^+$ T-cell stimulation in vitro and vivo.[86-88] Additionally, reduction-oxidation states can be exploited for timed payload delivery given that the endosomal compartment is reductive while the lysosomal compartment is increasingly oxidative, compared to an extracellular environment that is only mildly oxidative.[44,89] This concept has been utilized to deliver contents to the early endosome using reduction-sensitive materials,[91] while oxidation-sensitive particles have been used to generate humoral and cellular immunity to particle-loaded ovalbumin.[92] Furthermore, biomaterial schemes have been developed to facilitate endosomal escape through use of these same endosomal triggers of pH and reduction state to generate membranolytic products with demonstrated ability for antigen delivery and cross-presentation.[91,93]

Finally, reports indicate that particle size can modulate tissue targeting, cellular uptake specificity and adjuvancy. For example, despite a presumed loss in phagocyte-targeting specificity, subcutaneous injection of very small sized nanoparticles (20–45 nm) have been shown to be driven to lymph nodes through interstitial fluid flow and taken up by lymph node-resident DCs.[92,94-96] Additionally, it has been reported that 40–100 nm inert particles injected subcutaneously were taken up more efficiently by DCs, while 1 μm particles were taken up more by macrophages, and that 40 nm antigen-loaded particles were more immunogenic.[96] On the other hand, phagocyte targeting has been shown to also be dependent on the route of administration, as intraperitoneal injection of PLGA microparticles favored the uptake and migration to lymph nodes by macrophages while subcutaneous injection favored DCs.[39] PLGA microparticles size (a range of 1–14 μm) has been demonstrated to direct adjuvancy of antigen-loaded PLGA microparticles delivered orally, with an optimal particle size of 4 μm.[97] Interplay between the parameters of particle size and receptor targeting can also be a factor, receptor-targeted nanoparticles (0.2 μm) but not microparticles (2 μm) have demonstrated increased specificity of antigen delivery to DCs, in vitro.[98]

Dendritic Cell Receptor Targeting

The targeting of antigens to specific DC receptors has been investigated primarily utilizing soluble antigens. Normally, endocytosed soluble antigens are cross-presented by DCs less efficiently than particulate antigens which are taken up by phagocytosis,[99] however, DC receptor-targeting has been shown to facilitate cross-presentation.[100,101] This is an important consideration given that cross-presentation by DCs can enable peripheral $CD8^+$ T-cell

cross-tolerance.[102] Ligation of antigens to molecules targeting DC upatke receptors, for example, the dendritic and epithelial cell 205 receptor (DEC-205,[103]) can increase uptake efficiency by approximately 100-fold.[101,104,105] Approaches for targeting DC receptors have generally involved either natural receptor ligands or the use of antibodies raised against specific receptors. Ligands for DC receptors that have been investigated for soluble antigen delivery include heat shock proteins,[106] bacterial toxins,[107] sugar residues[108] and CD40-ligand.[109] For example, targeting C-type lectin receptors has been accomplished through incorporation of specific sugar residues such as mannose/mannan.[110] Additionally, antigen targeting to DC receptors through the use of receptor-specific antibodies has been widely investigated.[108] Dendritic cell surface receptors which have been antibody-targeted for antigen delivery include the integrin CD11c/CD18,[111] Fc receptors,[112,113] and the C-type lectin receptors: mannose receptor,[114,115] DEC-205,[100,116] and DC-SIGN.[117,118]

Notably, immunogenic applications benefit from targeting antigen to a receptor which provides concomitant DC activation (e.g., CD40-ligand[119]), however a tolerogenic application will require targeting DC receptors which do not activate DCs. Fortunately, certain receptors have the potential for DC targeting reportedly without activation. For example, targeting soluble antigen to DEC-205 did not activate DCs, and in fact, lead to DC-induced peripheral T-cell unresponsiveness in the absence of an additional stimulatory signal.[101,120,121] In fact, targeting antigen to DEC-205 has demonstrated the ability to induce tolerance in animal models of T1D.[122,123] Additionally, inhibitory receptors such as the Fc receptor, CD32b,[124,125] or DC inhibitory receptor-2 (DCIR2/Clec4a2)[126,127] may present a potential way to simultaneously target and provide inhibitory signals to DCs.

A number of these targeting approaches described for soluble antigen delivery have also been applied toward DC-targeting of microparticles. For example, mannose/mannan has also been surface bound to microparticles, demonstrating a dose-dependent response in DC uptake.[128] In this study, Wattendorf et al. also investigated targeting microparticles to DC integrins by incorporating the RGD adhesive peptide. Surface-modified microparticles with RGD peptide also increased DC uptake (this aspect is corroborated by others[129]) and combining both mannan and RGD provided cumulative effects on microparticle uptake by DCs. It was also found that these ligand-modified microparticles did not activate DCs, as determined by expression of MHC-II, CD86 and CD83.[128] Furthermore, microparticles have been surface modified to target DEC-205,[86,98] demonstrating increased receptor-mediated uptake by DCs, migration to lymph nodes and stimulation of naïve T-cells in vivo.[86] Additionally, other microparticle modification approaches to target DCs include surface-tethering antibodies targeting CD40, and the $\alpha_V\beta_3$, $\alpha_V\beta_5$ and CD11c integrins, reporting differential levels of DC activation for each.[130,131] This is an informative comparison, as the functional consequences of DC integrin binding is not well understood,[132,133] and are currently under investigation.[134]

Incorporating Immunomodulatory Signals into Microparticles

Immunogenic applications of microparticle-based vaccines have established precedent for the incorporation of immunomodulatory factors. In particular, incorporation of adjuvants in polymeric microparticles have ranged from the well-established (e.g., alum[135]) to the well-defined (e.g., toll-like receptor ligands: CpG,[136,137] poly(I:C),[87] monophosphoryl lipid A[138,139]). Within these types of combined antigen/adjuvant microparticle systems, co-encapsulation has been shown to be more beneficial than co-administration, by sustaining immunomodulatory signaling after antigen collection.[136,140] Notably, the use of polymeric microparticles can facilitate a mechanistic understanding of DC endocytic processes. For example, the requirement that both antigen and TLR ligand need to be colocalized in the same endosome in order distinguish non-self-antigen from self antigen has recently been determined.[141] Together, these data begin to reveal to a set of design principles for immunogenic microparticle-based vaccine applications. Whether or not these design principles will have an effective tolerogenic/immunosuppressive counterpart remains to be seen.

In addition to stimulatory signals, a promising approach is the incorporation of factors such as chemokines and growth factors to both attract DCs and condition the local injection microenvironment. For example, chemokines such as the peptide N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys,[142] macrophage inflammatory protein-3α (MIP-3α; CCL20),[142,143] CCL19,[144] CCL21,[144] and granulocyte macrophage colony-stimulating factor (GM-CSF)[145,146] have all been investigated in biomaterial timed-release systems consisting of microparticles and/or hydrogels, demonstrating sustained ability to attract DCs. GM-CSF is a particularly interesting case, as use has been FDA-approved, and its effects are pleiotropic by: serving as a chemoattractant for both DCs and monocytes, promoting monocyte differentiation into DCs and increasing DC endocytic activity.[145,146] Furthermore, treatment with GM-CSF in NOD mice has been shown to be able to delay T1D through acting on DCs and expanding Foxp3$^+$ Tregs.[147]

Compared to investigations with stimulatory molecules, incorporation of immunosuppressive molecules into microparticles has only recently begun to be explored. In particular, incorporation of rapamycin-loaded microparticles targeted to DCs has demonstrated generation of DCs maintained in an immature state,[148-150] that resist maturation,[150] with lowered expression of ICAM-1,[149] with high levels of TGFβ1 produced,[150] and which have reduced T-cell stimulatory capacity.[148,150] Additionally, microparticles with encapsulated IL-10 plasmid targeted to DCs generates DCs which elicit only weak T-cell stimulatory capacity,[151] and which expand Foxp3$^+$ Tregs.[152]

Dendritic Cell-Targeting Microparticle-Based Vaccine for T1D

Other types of particle-based approaches have been explored, such as the recent investigation using T-cell-targeting nanoparticles

with tethered peptide-MHC molecules.[153] However, there is apparently only one study which has reported DC-targeting microparticle-based vaccines for T1D amelioration, led by the Giannoukakis group at the University of Pittsburgh School of Medicine. This line of research began with the demonstration that injection of NOD DCs treated ex vivo with antisense oligonucleotides knocking down expression of the costimulatory molecules CD40, CD80 and CD86, was able to significantly delay the incidence of diabetes in NOD mice.[154] This was subsequently linked to the increased prevalence of regulatory $CD4^+CD25^+$ T-cells through DC production of IL-7.[155] Remarkably, this approach is currently being investigated in a phase I clinical trial, in which antisense-treated autologous DCs are being administered to established T1D adult patients in order to establish safety (ClinicalTrials.gov Identifier: NCT00445913). Despite the promise of this study, anticipating the limitations of cell-based vaccinations, the Pittsburgh group is also pursuing a microparticle-based vaccine for T1D targeting DCs in vivo. This approach relies on the demonstrated ability[67] of microparticle-encapsulated antisense oligonucleotides to effect gene knockdown in DCs. In the study by Phillips et al. polymeric microparticles carrying antisense oligonucleotides to CD40, CD80 and CD86 were delivered to a subcutaneous site proximal to the pancreatic lymph nodes in NOD mice.[10] The injected microparticle formulation augmented $Foxp3^+$ Tregs and provoked hypo-responsiveness to β-cell antigen without compromising global immune responses to alloantigen. A fraction of the microparticles were found to accumulate primarily within the pancreatic lymph nodes and to a lesser extent the spleen after injection subcutaneously or intraperitoneally, indicating uptake and migration by phagocytes. Most importantly, the microparticle formulation both prevented and reversed new-onset T1D.[10] Notably, this formulation did not employ the use of a delivered antigen, which raises questions regarding the antigen-specific nature of the immune response. Additionally, it's not clear if the microparticle system used (ProMaxx[156]) is optimal for loading and extended delivery of antisense oligonucleotides, given that eight injections were necessary to effect reversal.

Concluding Remarks

Collectively, from these examples we can see that the advantages of microparticle-based vaccines delineated in the literature with immunogenic applications are beginning to be appreciated by the autoimmunity/immunosuppression community. Critically, the advantages of in vivo targeting of DCs/phagocytes, antigenic depot, incorporation of additional immunomodulatory factors and sustained release are all retained in these new applications, opening up a multitude of new avenues for research in T1D. Given the number of antigens, targeting molecules, immunomodulatory agents, chemokines and growth factors that may be of interest for delivery to DCs, the large number of potentially useful microparticle formulations is staggering. Advancement in key areas would enable transformative gains. Primarily, the identification of predictive in vitro markers is critical.[157] Additionally, development of a high-throughput screening methodology for the efficient, systematic examination of DC responses to microparticle formulations[45] is also important, representing a step toward personalized vaccines.

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