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Engineering Tolerance Using Biomaterials to Target and Control Antigen Presenting Cells

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Abstract: Autoimmune diseases occur when cells of the adaptive immune system incorrectly recognize and attack "self" tissues. Importantly, the proliferation and differentiation of these cells is triggered and controlled by interactions with antigen presenting cells (APCs), such as dendritic cells. Thus, modulating the signals transduced by APCs (e.g., cytokines, costimulatory surface proteins) has emerged as a promising strategy to promote tolerance for diseases such as multiple sclerosis, type 1 diabetes, and lupus. However, many approaches have been hindered by non-specific activity of immunosuppressive or immunoregulatory cues, following systemic administration of soluble factors via traditional injections routes (e.g., subcutaneous, intravenous). Biomaterials offer a unique opportunity to control the delivery of tolerogenic signals *in vivo* via properties such as controlled particle size, tunable release kinetics, and co-delivery of multiple classes of cargo. In this review, we highlight recent reports that exploit these properties of biomaterials to target APCs and promote tolerance via three strategies, i) passive or active targeting of particulate carriers to APCs, ii) biomaterial-mediated control over antigen localization and processing, and iii) targeted delivery of encapsulated or adsorbed immunomodulatory signals. These reports represent exciting advances toward the goal of more effective therapies for autoimmune diseases, without the broad suppressive effects associated with current clinically-approved therapies. [*Discovery Medicine* 21(117):403-410, May 2016]

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Introduction

Autoimmune diseases are conditions in which the immune system mistakenly attacks host molecules, cells, and tissues. These conditions impact both children and adults, with some of the most common diseases including multiple sclerosis (MS), type 1 diabetes, lupus, and rheumatoid arthritis. Generally speaking, autoimmunity occurs when immune tolerance -- the mechanisms the body uses to regulate healthy immune function -- fails. The control systems governing these processes are incredibly complex, involving integration of signals from cytokines, chemokines, soluble factors, stromal components, and cells, both at sites of disease and within the spleen and lymph nodes (LNs) -- organs that direct immune function. Normally, antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, survey peripheral blood and tissue and migrate to LNs and spleen after encountering bacteria, viruses, or other foreign pathogens. Once in these sites, pathogens are processed by APCs and the resulting antigen fragments are displayed on the cell surfaces in protein assemblies termed major histocompatibility complexes (MHC) (Mueller and Germain, 2009). Display of antigen in MHC, along with the appropriate "warning" or co-stimulatory signal, leads to activation of resident T and B cells that exhibit specificity for the same antigen being presented on the APCs (Morelli and Thomson, 2007; Pereira and Fraefel, 2015).

Because of the critical role DCs and other APCs play in generating adaptive immune response, APCs are important targets for both traditional prophylactic vaccines and for therapeutic vaccines aimed at cancer or autoimmunity (Andorko *et al.*, 2015a). In particular, many

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studies demonstrate the role of DCs in promoting tolerance through direct production of immunosuppressive cytokines, or by polarizing T cells and other populations that typically drive disease away from inflammatory phenotypes. For example, modulation of DC signaling can promote expansion of regulatory T cells (T_{REGS}) or other suppressive populations, as well as induce cell deletion and non-responsiveness ("anergy") (Maldonado and von Andrian, 2010; Manicassamy and Pulendran, 2011; Osorio *et al.*, 2015; Soroosh *et al.*, 2013). In the past decade, there has thus been an explosion of experimental therapies aimed at exploiting these capabilities for more potent and selective autoimmune therapies. Systemic delivery of rapamycin or other immunosuppressants has been used to alter DC phenotype in the context of transplantation, type 1 diabetes, lupus, and MS (Chalmers *et al.*, 2015; Salmond and Zamoyska, 2011; Taner *et al.*, 2005; Thomson *et al.*, 2009). However, these approaches are hindered by the systemic manner in which regulatory cues are delivered, echoing a nearly universal challenge facing preclinical and approved autoimmune therapies: broad suppression that leaves patients immunocompromised. Further, our understanding of the complex network of lymphatics that facilitate the migration of APCs, T cells and B cells -- for example, between LNs and peripheral sites of disease -- is still developing, suggesting new opportunities to study and target these pathways to promote tolerance (Aspelund *et al.*, 2015; Louveau *et al.*, 2015; Mankia and Emery, 2015). For example, two landmark papers in 2015 revealed new lymphatics that connect the central nervous system (CNS) -- the site of autoimmune attack during MS -- with deep cervical lymph nodes (Aspelund *et al.*, 2015; Louveau *et al.*, 2015). Together, all of these ideas underscore the potential for designing new tolerogenic therapies that provide more specific delivery to APCs and the tissues in which they reside, capabilities that biomaterials are uniquely-suited to provide. In this review, we discuss properties of biomaterials that can be exploited for targeting and controlling APCs, and highlight recent examples of how these materials are being used to promote tolerance in autoimmunity and transplantation. These strategies are grouped into three categories: i) passive or active targeting of particulate carriers to APCs, ii) biomaterial-mediated control over antigen localization and processing, and iii) targeted delivery of encapsulated or adsorbed immunomodulatory signals (Figure 1).

Biomaterials Offer General Features that Are Useful for Engineering Immune Response

From one perspective, biomaterials might be thought of as a broad collection of materials -- synthetic polymers, lipids, imaging agents, proteins, nucleic acids -- that become biomaterials when applied to biological questions or applications. These materials are commonly formulated into nanoparticles (NPs) or microparticles (MPs), organized into self-assembling structures such as nanostructured protein complexes or liposomes, or used to fabricate biocompatible scaffolds and devices. Many of the attractive features of biomaterials are particularly useful in drug delivery, vaccination, and immunotherapy (Andorko *et al.*, 2015a; Jones *et al.*, 2015; Shao *et al.*, 2015; Singh and Peppas, 2014). For example, lipid and polymer particles can be formulated with multiple cargos to achieve co-delivery, modified with ligands for improved targeting, designed with pro-

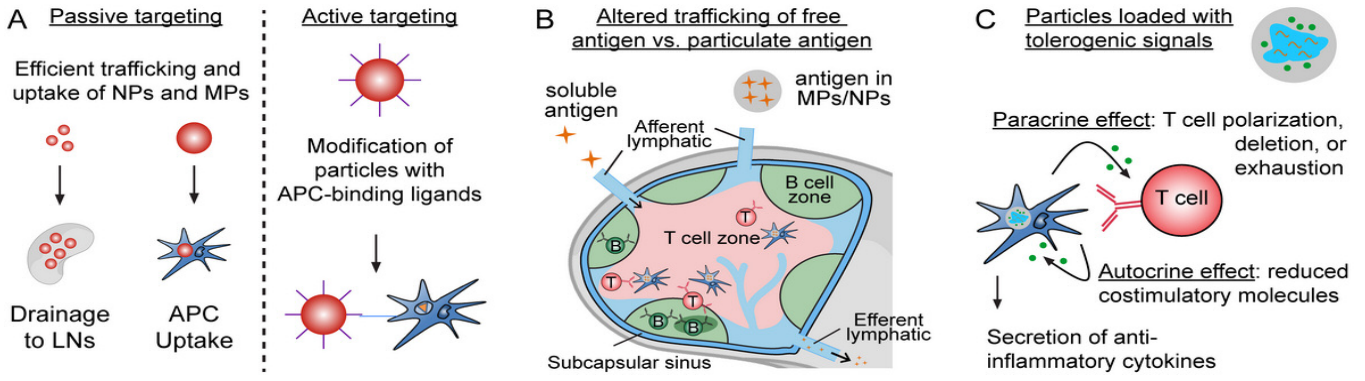

Figure 1. Strategies to exploit biomaterials in promoting immune tolerance. A) Biomaterials exhibit general features that can be exploited for passive and active targeting to achieve tolerance. B) Induction of tolerogenic function using biomaterials to alter trafficking and processing of self-antigens by APCs. C) Harnessing biomaterials to generate tolerance by targeting regulatory immune cues to APCs.

grammable stabilities for controlled drug release, and used to protect biologic cargo from enzymatic degradation or pH gradients. These features, along with the ability to tune physicochemical properties such as particle size, also provide many opportunities to passively or actively target APCs.

Passive strategies for targeting APCs

The size of NP or MP vaccine and immunotherapy carriers, such as those formed from polymers or lipids, has two major implications for interactions with APCs (Andorko *et al.*, 2015a; Irvine *et al.*, 2013). First, APCs have evolved to efficiently phagocytose particles over nanometer and micrometer size ranges. Thus, since most biomaterial vaccine carriers exhibit sizes from tens of nanometers to several microns, this characteristic provides an immediate advantage for recognition, internalization, and processing of antigens or other immune signals in the particles. Second, sub-100 nm NPs are able to drain to LNs following peripheral injection much more efficiently than MPs (Reddy *et al.*, 2007). To elicit potent adaptive responses, MPs are more reliant on trafficking by APCs from the injection site, illustrating the direct impact size has on the route and efficiency with which biomaterial carriers reach LNs. A number of studies have also revealed the importance of other physicochemical features such as shape and charge in immune cell interactions, so these aspects will likely create new levers which can be pulled to further encourage non-specific recognition and uptake of NPs and MPs by APCs (Foged *et al.*, 2005; Sunshine and Green, 2013; Sunshine *et al.*, 2014).

Active strategies for targeting APCs

Active targeting strategies for biomaterials have been studied by exploiting APC surface markers, as well as targeting APCs at the tissue level for improved LN delivery. Several reports, for example, have improved DC targeting using polymeric or lipid NPs displaying monoclonal antibodies specific for DEC-205, a characteristic transmembrane protein on DCs (Kwon *et al.*, 2005; van Broekhoven *et al.*, 2004). At the tissue level, one exciting recent approach involved co-opting a natural albumin shuttling network involved in trafficking proteins and other factors to LNs (Liu *et al.*, 2014). In this approach, lipids were designed with an albumin binding domain, peptide antigens, and inflammatory toll-like receptor agonists (TLRa) as adjuvants. TLRas bind receptors on APCs that have evolved to alert the immune system upon encountering molecular patterns or danger signals that are common in pathogens. Immunizing mice with the lipid conjugates produced striking antigen-specific immunity in several mouse models of cancer. Another approach focused on LNs is engineering of the LN microenvironment with MPs. In this work, immune signals were localized and retained in LNs through direct injection of degradable MPs that are too large for rapid drainage from LNs (Jewell *et al.*, 2011). This strategy can be used to rapidly generate large populations of antigen-specific T cells with direct control over the signals introduced to APCs and other resident cells. Several recent studies illustrate an interesting feature of polymeric biomaterials: many of these polymers can activate inflammatory immune pathways even in the absence of antigens or other adjuvants (Andorko *et al.*, 2015a; 2015b; Sharp *et al.*, 2009). With improved understanding, this is a feature that can be harnessed for both prophylactic and therapeutic applications. In tolerance specifically, the possibility of intrinsic immunogenicity that could exacerbate disease creates new motivation to develop materials that provide features of biomaterials (e.g., tunable size, co-delivery, targeting) without the risk of inherent inflammatory carrier properties. Some studies have worked to mask this intrinsic activity of a widely-used polymer, poly(lactide-co-galactide) (Lewis *et al.*, 2012), and several new technology platforms based on self-assembly of proteins or nucleic acids might also be useful to exploit for tolerance (Chiu *et al.*, 2015; Hudalla *et al.*, 2014; Zhang *et al.*, 2015).

All of the features presented thus far are of general relevance for vaccination and immunotherapy. Below we discuss specific examples in which biomaterials have been used to influence APCs in the generation and control of tolerance. These are divided into two strategies: i) use of biomaterials to change how self-antigens are trafficked or processed, and ii) use of biomaterials to deliver regulatory cues to APCs. Biomaterials are also being used in other interesting ways to induce tolerance -- for example, directly altering T cell phenotype -- that are presented in recent reviews and articles (Andorko *et al.*, 2015a; Hlavaty *et al.*, 2015; Northrup *et al.*, 2015; Tsai *et al.*, 2010).

Biomaterials Can Be Used to Alter the Trafficking and Processing of Self-antigens

Our understanding of the pathology of many autoimmune diseases has increased remarkably in the past several decades, allowing identification of some key self-antigens in MS and type 1 diabetes, and emerging candidates for other diseases such as lupus and rheumatoid arthritis (Bluestone *et al.*, 2015; Lutterotti and Martin, 2014). In the subsections below, we discuss two strategies to promote tolerance by altering the trafficking or processing of these self-antigens.

Altered trafficking of self-antigen to exploit debris clearance pathways

MS is one of the most-studied autoimmune diseases. Unsurprisingly then, several biomaterial strategies aim to generate myelin-specific tolerance to stop the attack of myelin -- the molecule attacked in the CNS during MS -- without the non-specific immunosuppression of existing therapies. Building on earlier work using splenocytes modified to display self-peptides, Shea, Miller, and colleagues have developed a strategy for tolerance involving covalent modification of polymeric MPs with myelin peptides (Getts *et al.*, 2012; Hunter *et al.*, 2014). In these reports, both prophylactic and therapeutic treatment with myelin proteolipid protein (PLP) generated efficacious tolerance during a mouse model of relapsing remitting MS, relapsing remitting experimental autoimmune encephalomyelitis (RR-EAE). A cardinal finding of this work reveals that conjugation of PLP to the MPs leads to trafficking of peptide to cells in the marginal zone of the spleen that are positive for the Macrophage Receptor with Collagenous Structure (MARCO), a scavenger receptor involved in debris clearance. Although the exact mechanism is still being investigated, a working hypothesis suggests upregulation of MARCO and localization to $MARCO^+$ cells improves antigen presentation, which, in the absence of other co-stimulation, might promote regulatory responses. Along these lines, functional reduction in RR-EAE severity was accompanied by increased T_{REGS}, reduced pathogenic T cell infiltration into the CNS, and anergy. This general approach has also recently been employed to improve graft survival in a pre-clinical mouse transplant model (Bryant *et al.*, 2014).

Along with MARCO, the body uses many additional pathways to clear apoptotic cells and other debris. Interestingly, antigens displayed in these milieus can nucleate antigen-specific tolerance. One of the hypotheses underlying this outcome is the possibility that the presence of antigen in the absence of other inflammatory signals or a non-activating environment drives deletion of T cells or promotes anergy (Griffith and Ferguson, 2011). The Hubbell lab has exploited this idea by conjugating antigens to erythrocytes, a cell population with a large number of apoptotic events and recycling on a daily basis. (Grimm *et al.*, 2015; Kontos *et al.*, 2013). These studies reveal that compared with free antigen, antigen bound to glycophorin A on erythrocytes was present in a much higher frequency of DCs, macrophages, and other APCs in the spleen and liver. This uptake, along with the absence of co-stimulation, was shown to promote T_{REGS} and to drive antigen-specific deletion of $CD4^+$ and $CD8^+$ T cells through increased PD-1 signaling, a natural negative regulator of immunity. Functionally, erythrocyte binding of a candidate antigen associated with disease in diabetes prevented onset of hyperglycemia in a mouse model of type 1 diabetes. Another approach along this same theme is driven by the intriguing idea that even particles that do not include self-antigen might help promote tolerance by exploiting apoptotic clearance mechanisms to trigger deletion or anergy. In this report, infusion of MPs formed from either degradable or non-degradable polymers induced tolerance in mouse models of myocardial infarction, EAE, colitis, peritonitis, and lethal flavivirus encephalitis (Getts *et al.*, 2014). This surprising result required the particles to exhibit a negative charge. Although the mechanism needs further study, negatively-charged MPs seem to be internalized by $MARCO^+$ inflammatory monocytes, leading to apoptosis of these cells instead of trafficking to sites of inflammation and autoimmune attack. Together these strategies underscore the link between trafficking of self-antigens or other particulates with the inter-connected regulatory pathways that cooperate to promote tolerance.

Disruption of interactions between APCs and T cells

In addition to altered antigen trafficking, several recent studies have exploited biomaterials to promote tolerance by blocking interactions between APCs and T cells that are required for pro-immune adaptive immunity. The Berkland lab used a hyaluronic acid backbone to graft this polymer with myelin epitopes and peptides that bind the B7 (CD80/CD86) protein on APCs (Northrup *et al.*, 2014). This is an important pathway during APC/T cell interactions, thus mice receiving a three-injection regimen of these polymers to block B7 signaling exhibited attenuated disease during RR-EAE. Other strategies have focused on blocking pro-inflammatory T cell activation by directly targeting T cells. In one approach, NPs were functionalized with MHC complexes displaying peptide epitopes attacked during type 1 diabetes (Tsai *et al.*, 2010). These studies reversed autoimmunity in mouse models of type 1 diabetes, functioning through a proposed mechanism in which low, sustained stimulation of antigen-experienced $CD8^+$ T cells is generated by the NPs. This presentation without co-stimulation drives a small population of memory-like, regulatory $CD8^+$ T cells that control disease in an antigen-specific manner.

Targeting Regulatory Cues to APCs via Biomaterials Can Be Used to Activate Tolerogenic Processes

The strategies discussed in the previous section relied on manipulating how antigens are received by the

immune system to activate regulatory processes. An exciting parallel set of approaches is based on changing the response to self-antigens using biomaterials to control the delivery of regulatory signals either with self-antigen, or as monotherapies that alter response to self-antigens presented in LNs (Andorko *et al.*, 2015a; Northrup *et al.*, 2015). Some of these have targeted T cells with suppressive drugs to reduce inflammation, limit self-reactive effector T cells (e.g., T_H17, T_H1), and drive regulatory populations such as T_{REGS}. Maldonado *et al.*, for example, recently reported a robust approach to induce tolerance using MPs loaded with self-antigens and rapamycin, a drug that promotes regulatory functions in both T cells and DCs (Maldonado *et al.*, 2015). In this report, particles loaded with antigen created only a modest effect, whereas co-loading with rapamycin drove efficient tolerance in several mouse models, including RR-EAE and hemophilia A. Importantly, tolerance was antigen-specific. This approach, coupled with those in the previous section involving only antigen highlight important open questions: What is the role of self-antigen delivery in promoting tolerance? Why does addition of suppressive drugs drive synergistic effects when drug alone has significantly reduced impact? Answers to these questions will help inform clinical translation by revealing the combinations of signals that should be delivered, what tissues (e.g., LNs, disease sites) should be targeted, and the regimens or kinetics over which delivery should occur.

Biomaterial-mediated delivery of drugs that suppress pro-immune APC functions

Because of the importance of DCs and other APCs in tolerance, biomaterials have recently been combined with a number of different drugs and suppressive immune signals to specifically target APCs. Rapamycin, as mentioned above, is a common immunosuppressant that can polarize the phenotypes of APCs to secrete regulatory cytokines, promote T_{REGS}, and reduce activation (Salmond and Zamoyska, 2011). In one of the earlier reports in the field, the Little lab used MPs to solubilize and deliver rapamycin to primary DCs, leading to reduced ability of these cells to activate T cells *in vitro* (Jhunjhunwala *et al.*, 2009). Micelles have also been used to encapsulate rapamycin and analogs of this drug to reduce maturation and activation of DCs in draining LNs after injection (Dane *et al.*, 2011). These effects improved tail allograft survival in a mouse transplant model. Dexamethasone, another immunosuppressant, has also been used toward similar goals in EAE by encapsulation with myelin peptides in acetylated dextran MPs (Peine *et al.*, 2014).

Biomaterial-mediated delivery of drugs that enhance APC regulatory pathways

The reports just described focus on suppressing stimulatory immune functions in APCs, but immunosuppressants and other signals can also enhance regulatory pathways, or in some cases modulate both routes to enhance tolerance. Mycophenolic acid, for example, is a classic immunosuppressant. The Goldstein and Fahmy labs have used NPs loaded with this drug to prolong survival of skin allografts in mice (Shirali *et al.*, 2011). One of the interesting features of this approach is the finding that efficacy could be achieved at concentrations 1,000-fold lower than those required using soluble MPA. Mechanistic studies revealed MPA NPs upregulated the natural regulatory functions of PD-L1 signaling on DCs. In a subsequent study, injectable nanogels were designed by complexing MPA in cyclodextrin complexes (Look *et al.*, 2013). These assemblies were then loaded into degradable NPs and crosslinked to form a gel that sustains the release of MPA. After either prophylactic or therapeutic treatments, gels reduced inflammatory cytokines and increased survival during murine systemic lupus erythematosus (SLE) -- a common pre-clinical lupus model. Another recent approach combined two different particle sizes to target self-antigens to DCs in smaller, phagocytosable MPs while delivering signals that promote a tolerogenic milieu in larger, non-internalizable MPs (Lewis *et al.*, 2015). The smaller MPs were loaded with vitamin D3 and diabetes antigens and mixed prior to injection with larger MPs encapsulating transforming growth factor beta 1 (TGF-β1) and granulocyte-macrophage colony-stimulating factor (GM-CSF). In this report, treatment prevented the onset of type-1 diabetes in 40% of mice. Interestingly, DCs and other APCs from treated mice also exhibited increased Gr-1, an upstream indicator of cells with the potential to differentiate into tolerogenic APCs subsets.

Several other approaches have used small molecule drugs and ligands to enhance specific regulatory pathways. The Quintana lab has worked extensively with the aryl hydrocarbon receptor, a transcription factor present in DCs and other APCs that promotes T_{REGS} upon engagement. These investigators adsorbed a ligand for this receptor to gold NPs and used these particles, particles loaded with myelin peptide, or particles loaded with both signals to treat cells and mice. NP formulations containing both the ligand and myelin peptides induced tolerogenic DCs in culture, as well as in mice with EAE, and increased T_{REG} frequencies. These effects reduced disease severity, but required the presence of myelin in the NPs, suggesting an antigen-spe-

cific characteristic to the tolerance.

Another exciting new approach is based on controlling the metabolic activity of DCs to alter the interactions of these cells with T cells. In this report, Gammon *et al.* developed NPs loaded with a metabolic modulator of glutamate metabolism, N-Phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxamide (PHCCC) (Gammon *et al.*, 2015). DCs release glutamate during inflammation, and this molecule is processed and metabolized by the metabotropic glutamate receptor family. The specific receptors that metabolize glutamate help control the balance between inflammatory and regulatory DC function. In primary cell culture, NPs loaded with PHCCC were 36-fold less toxic then soluble PHCCC and dramatically reduced DC activation. During co-culture, these effects polarized T cells toward T_{REGS} while reducing inflammatory cytokines and phenotypes. Subsequent studies in EAE demonstrated that sustained release of PHCCC from NPs delayed disease onset and severity at doses and intervals where soluble drug had no effect. Together, these studies suggest a new strategy to promote tolerance by altering APC/T cell interactions through programming of metabolic function. The excitement of this idea is underscored by recent discoveries that reveal new connections between metabolism and immune response (Wang *et al.*, 2015; Buck *et al.*, 2015).

Conclusion

The examples discussed here illustrate the exciting potential of biomaterials to generate immune tolerance that is more specific and more potent. Thus far, most of the work in this area has been pre-clinical, so multi-disciplinary teams should support continued advancement of these technologies toward the clinic. Similarly, communication between the engineering and immunology disciplines is critical to ensure clinically-relevant problems are being attacked, and that the appropriate technologies are being deployed. The coming years are also sure to bring excitement in emerging areas where tolerance and biomaterials can be used. Some of these include regenerative medicine -- for example in diabetes and transplantation, in immunometabolism where new links are being discovered between lymphocyte phenotype and function (Wang *et al.*, 2015), and in organ-specific autoimmune-disease as new connections are made between sites of self-attack and the local microenvironment of LNs and the spleen.

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Disclosure

The authors declare no competing interests.

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