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Targeted Immunomodulation Using Antigen-Conjugated Nanoparticles

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Abstract

The growing prevalence of nanotechnology in the fields of biology, medicine and the pharmaceutical industry is confounded by the relatively small amount of data on the impact of these materials on the immune system. In addition to concerns surrounding the potential toxicity of nanoparticle (NP)-based delivery systems, there is also a demand for a better understanding of the mechanisms governing interactions of NPs with the immune system. Nanoparticles can be tailored to suppress, enhance, or subvert recognition by the immune system. This “targeted immunomodulation” can be achieved by delivery of unmodified particles, or by modifying particles to deliver drugs, proteins/peptides or genes to a specific site. In order to elicit the desired, beneficial immune response, considerations should be made at every step of the design process: the NP platform itself, ligands and other modifiers, the delivery route, and the immune cells that will encounter the conjugated NPs can all impact host immune responses.

INTRODUCTION

Nanomaterials were reportedly first alluded to as “molecular machines” in 1959 by Nobel Prize winner Richard Feynman. Fifteen years later, Norio Taniguchi coined the term “nanotechnology”. By 1996, the first conference in nanobiology took place, and the use of nanoparticles for the advancement of biomedicine was on the forefront of scientific research. [1] Today, the applications for nanotechnology in biomedicine continue to broaden in scope, encompassing drug delivery, diagnostic imaging, vaccine development and tissue regeneration. The majority of research concerning the use of nanoparticles as vehicles for antigen delivery focuses on encapsulated antigens. Here, we discuss the considerations,

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advantages and disadvantages of using various types of nanoparticle platforms for antigenic display on the particle surface, and the types of immune responses those various formulations can provoke.

FACTORS AFFECTING NANOPARTICLE RECOGNITION AND UPTAKE BY APCs

The induction of T cell-mediated immunity requires the presentation of antigen to T cells by mature and activated dendritic cells (DCs) *in vivo*.[2,3] The ability of DCs to promote T cell activation and differentiation is dependent upon multiple factors, including 1) antigen-capture, 2) DC maturation, and 3) the efficient processing and presentation of antigen to T cells. Under steady-state conditions, tissue resident DCs are quiescent, constantly surveying the environment for antigens to acquire and present to T cells. Under these conditions, the functional outcome of DCs presenting self-antigens to T cells is the maintenance of peripheral tolerance through anergy or deletion.[4] However, the acquisition of foreign antigen by DCs in the presence of inflammatory signals elicited by cytokines such as TNF-α and IL-1β[5], the ligation of TLRs and other pattern-recognition receptors,[6] or tissue injury and cell necrosis[7] are critical steps in the activation and functional maturation of dendritic cells. These signals induce phenotypic and morphological changes in dendritic cells and their precursors that facilitate their migration to the lymph nodes via the CCR7-CCL19/21 axis[5] where they are able to initiate contact with a dense number of T cells,[8] allowing for the priming of an effective T cell response. Ultimately $CD4^+$ and $CD8^+$ T cell activation requires multiple signals supplied by activated DCs (Figure 1) – 1) display of processed antigenic peptides in the context of major histocompatibility complex class I and class II molecules which are recognized by the T cell antigen receptor (TCR); 2) delivery of APC-derived costimulatory signals (*e.g.*, CD80 or CD86) which interact with receptors (*e.g.*, CD28) on naïve T cells; and 3) delivery of cytokines (*e.g.* IL-12, IL-4, IL-1/IL-6/IL-21, and TGF-β) which instruct the differentiation of naïve T cells to become T_H1, T_H2, T_H17 or iTreg effector cells, respectively.[9] Since viruses and tumors have developed mechanisms that evade recognition by the innate immune system, and therefore escape elimination by the adaptive immune system, nanoparticle vaccine strategies that effectively target and activate dendritic cell pathways are an area of intense interest. As such, multiple parameters of nanoparticle fabrication have been manipulated to enhance targeting of the vaccine-bearing nanoparticles towards DCs. Indeed, the chemical composition of nanoparticles can inform such inherent characteristics as size, solubility, and charge, which in turn can dictate their applicability as biocompatible agents, as can the route by which they are administered.

Route

Classically, antigen delivered subcutaneously leads to the induction of immunity while antigen delivered intravenously leads to the induction of immunological tolerance. Strategies designed to elicit immunity with nanoparticle delivery platforms have targeted similar routes of immunization with suboptimal effect: nanoparticles administered subcutaneously or intradermally may be taken up by tissue resident APCs or their precursors including monocytes, macrophages, and DCs; due to the low frequency of DCs in peripheral tissues, antigens delivered subcutaneously via nanoparticle platforms may not be the most efficient

method for priming optimal T cell responses. Indeed, observations from a study utilizing 0.5–1.0 μm latex or polystyrene particles delivered intracutaneously suggest that inflammatory blood-derived monocytes that were recruited to the site of particle administration are the dominant phagocytic population present in the skin during inflammation.[10] Interestingly, the particle-bearing monocytes were demonstrated to migrate to the draining lymph nodes where they differentiated into $CD11c^{low}$MHC class II^{high} DCs that were capable of priming allogeneic T cell responses to a similar capacity as the lymph node resident population of DCs. Nonetheless, the monocyte derived DCs comprised only 2.0% of the DC population, and only 0.04% of the total cell population in the draining lymph node. Compared to the number of LN resident $CD11c^{high}$ DCs, this monocyte-derived DC population represents an insignificant number of cells. Due to this observation, many strategies are being developed that target NP vaccines directly to the LN where they can interact with LN resident DCs.

Nevertheless, the skin, lungs, gut and LN are common tissue targets for the delivery of immunomodulatory agents, due to the presence of large numbers of immune cells and because the magnitude and duration of the immune response can differ depending on which tissue was targeted[11,12]. In addition to the use of targeting molecules on the surface of particles, the route of administration can help direct materials to the desired tissue compartments. The mucosal immune system is highly compartmentalized, and while immunization by parenteral routes does not induce mucosal antibodies, delivery by the mucosal route can induce both mucosal and systemic antibodies.[13] For example, pluronic-stabilized polypropylene sulfide (PPS) nanoparticles conjugated to ovalbumin induced cytotoxic T cell responses in the lung as well as remotely in the spleen following intranasal and pulmonary administration, indicating the induction of both systemic and mucosal immune responses.[14,15] Co-conjugation of ovalbumin and flagellin, a TLR5 ligand, enhanced humoral and cytotoxic T cell responses in mucosal airways, as well as remotely in the vaginal and rectal mucosal compartments when administered intranasally.[15] In contrast, following intradermal injection, these same particles were shown to preferentially traffic to the lymph node, and although both the cellular and humoral arms of the immune system were activated, these responses were not noted in mucosal compartments.[16] Lastly, pulmonary immunization with PPS nanoparticles expressing surface-linked tuberculosis antigen was reported to induce systemic T_H17 responses while intradermal administration of the same PPS nanoparticle conjugates did not induce any detectable T_H17 response, suggesting that the route can also dictate the type of T cell mediated immune response that is formed.[17]

Size and Shape

Nanoparticles can also be preferentially targeted to lymph node resident DCs simply by modifying the size of the nanoparticles. Smaller nanoparticles (20–40nm in size) are able to cross tissue barriers and traffic directly to the lymph node via interstitial flow, whereas larger nanoparticles (>100nm) require uptake by APCs to access the lymph node.[18] For instance, an investigation by Makino *et al*. confirmed that larger polystyrene particles 1.0 μm in diameter were preferentially phagocytosed by alveolar macrophages whereas smaller particles (0.2 μm)were not phagocytosed.[19] Uptake by macrophages in this study was also

dependent on additional surface properties including charge and "softness" of the particle, emphasizing that no single property determines particle fate, but rather that nanoparticle size, shape, composition, and other physical and chemical properties *together* will define the biological effect of nanoparticles. Fifis *et al*. compared particle size within both a viral (<0.5 μm) and a bacterial (>0.5 μm) range, and examined antigen uptake efficiency and *in vivo* localization of antigen-decorated carboxylated polystyrene particles in the lymph node (LN) after injection. They showed that lymph node-resident DCs most efficiently took up particles in the viral range, between 20 and 100 nm in diameter (optimally 40 nm).[20] Importantly, this "nanovaccine" elicited both antibody and CD8 T cell immune responses comparable to the adjuvants alum and monophosphoryl lipid A, and cleared established tumor masses in mice within 2 weeks after a single injection. A different study using 25nm PPS nanoparticles observed that up to 50% of the lymph node resident DCs were positive for the nanoparticles 24 hours after intradermal administration. In contrast, less than 10% of the lymph node DCs were positive for the co-injected, 100 nm sized PPS nanoparticles. Furthermore, the 25 nm PPS nanoparticles were capable of promoting the expansion of CFSE-labeled OT-II $CD4^+$ T cells conjugated to the model antigen ovalbumin, which subsequently led to induction of both cytotoxic and humoral immune responses.[16]

Interestingly, a study by Champion *et al*. reported that while size of the nanoparticle determined the ability of the APC to complete phagocytosis, it was the shape of the biomaterial that mediated the initiation of phagocytosis. Alveolar macrophages initiated phagocytosis of polystyrene particles of varying sizes *and* shapes, however shape was the sole determinant of whether the particle was successfully ingested.[21] A separate report also showed that ligand-coated gold NPs of similar size, charge, and hydrophobicity could either penetrate the plasma membrane without disruption or be trapped by endosomes depending on their homogenous versus random surface structure, respectively. Indeed, these NPs were designed as chemical isomers on a nanoscale, differing only in the molecular arrangement of surface chemical groups. Together, these studies indicate that shape and surface structure play a role in determining the uptake and subcellular fate of nanoparticles.[22]

Charge

Manipulation of nanoparticle surface properties can also modulate how the conjugated particles react with the immune system. Surfactants such as polyethylene glycol (PEG), phenylethylmalonamide (PEMA) and polyvinyl alcohol (PVA) can alter the NP's charge and solubility, thereby modifying overall biocompatibility. Attaching hydrophilic polymers (PEG, for example) to the NP surface greatly increases its solubility and can protect attached proteins from enzymatic degradation, thereby allowing enhanced delivery of the drug or protein of interest to the target population.[23] "Pegylated" NPs can also be used as platforms for lipophilic molecules, wherein insoluble molecules can be attached or adsorbed to the hydrated NPs.[24]

Surfactants are also commonly used to alter the charge of the NP. Charge can influence a nanoparticle's ability to navigate through biological barriers, including the stringent blood brain barrier (BBB).[25,26] Lockman *et al.* showed that in nanoparticles comprised of emulsifying wax and Brij 78 (a common nonionic surfactant): 1) the NP surface could be

correspondingly changed by using neutral, anionic, or cationic surfactants, and 2) while BBB integrity was not affected by neutral or low concentrations of anionic NPs, an immediate toxic effect and disruption of the BBB was caused by cationic NPs or high concentrations of anionic NPs. Further investigation of NP uptake in the brain demonstrated that thiamine-coated nanoparticles facilitated binding and/or association with BBB thiamine transporters over uncoupled particles, introducing a viable mechanism for nanoparticle ligand-mediated drug delivery to the brain.[26]

Upon intravenous injection, charged particles can either prevent or enhance the adsorption of serum proteins and opsonins. Coating nanoparticles with hydrophilic polymers has been shown to prolong their half-life by decreasing opsonization that results in rapid nanoparticle clearance by macrophages.[27–29] For example, an enhanced negative charge on PVA-coated PLG NPs affected recognition by APCs.[29,30] In contrast, dendrimer particles with positively charged surface amine groups were significantly more cytotoxic than their anionic counterparts.[31] Moreover, the charge of the nanoparticle can have a direct effect on antigen processing once captured by the intended APC population. Acid-degradable cationic OVA-encapsulating NPs composed of acrylamide and the amine monomer AETMAC were shown to increase the delivery of antigens into the MHC class I cross-presentation pathway of bone marrow-derived DCs at lower concentrations when compared to acid-degradable nanoparticles bearing a neutral charge,[32] and hepatitis C virus DNA adsorbed to the surface of cationic PLG nanoparticles was demonstrated to induce both cytotoxic T cell responses and seroconversion in treated animals.[33] The potent ability of cationic nanoparticles to deliver antigen to the MHC class I loading pathway was suggested to be the result of endosome disruption by the phagocytosed NP, and leakage of the antigen into the cytosol where it could then interact with the cross-presentation machinery.[34] Surface charge has also been implicated in NP distribution between the vascular and extravascular compartments of tumors.[35] Finally, increased charge may also influence the size and structure of conjugated NPs, resulting in NP-antigen-NP conjugates and potential agglomeration.[36]

One method that utilizes the surface charge of a platform to enhance vaccine delivery to DCs is the conjugation of DC-specific receptor antibodies to the surface of the nanoparticle. Use of a chemical cross-linker such as 1-(3-dimethylamiopropyl)-3-ethylcarbodiimide hydrochloride (ECDI) can be used to covalently link the antibody of interest to the free amines or carboxyl groups present on the surface of the nanoparticle. The conjugation of anti-DEC205 and anti-CD11c antibodies to PLG nanoparticles has been demonstrated to enhance nanoparticle uptake by DC and macrophage-mediated phagocytosis by as much as 50% *in vitro* and *in vivo*.[37] Similar effects on particle uptake have also been reported in a model using an ICAM-1 specific peptide conjugated to PLG nanoparticles in a HUVEC culture system.[38] However, such methods have been reported to have a non-stimulatory effect on DCs, even when injected into the footpad of mice,[37] and may therefore require additional factors to promote DC maturation and activation where protective immunity is the desired outcome. Thus, nanoparticle platforms for vaccine delivery may require the incorporation of danger signals into their design for the optimal induction cell mediated immunity.

Protein Corona

The exposure of nanoparticles to biological fluids such as blood, serum, and interstitial fluid leads to the formation of the protein corona, a layer of serum proteins and other biological factors that adsorb to the surface of the nanoparticle. Formation of the protein corona has become a topic of significant interest to investigators, as the corona is known to affect nanoparticle distribution, recognition, and uptake in biological environments.[39] The type of proteins that constitute the protein corona is largely dependent on the same factors that determine nanoparticle uptake by the immune system, such as charge, shape, structure, and size. One study observed that while the protein composition of the corona surrounding 100 nm and 50 nm neutrally-charged polystyrene particles shared 80% homology, protein homology was at 50% when the corona of negatively-charged 100 nm and 50 nm polystyrene particles where compared. Furthermore, the same study reported that 50 nm polystyrene particles of positive, neutral, and negative charge shared only 40% protein homology in their coronas, suggesting an important role for both charge and size in determining the composition of the protein corona.[40] Previous models of the protein corona suggested that the corona was composed of 2 layers: 1) the 'hard' corona which contains up to 100 proteins that become irreversibly adsorbed to the nanoparticle as a function of time, and 2) the 'soft' corona that is hypothesized to be in a state of dynamic exchange with free proteins in the serum as a result of binding kinetics. However, a recent study by Stauber and colleagues suggest that this model may require revision. The authors observed that the protein corona surrounding silica and polystyrene NPs was rapidly formed within less that 1.0 minute, was composed over 300 proteins, and that the composition of the protein corona changed qualitatively little as a function of time. Rather, the amount of a given protein adsorbed to the nanoparticle fluctuated between the early and late corona, and that significant changes to the corona were most likely to occur within 2 minutes of its formation. Thus, the stable composition of the corona, and its rapid development call into question the existence of a dynamic 'soft corona'.[41] Moreover, the authors reported that the proteins making up the corona had a net negative charge irrespective of the surface charge of the nanoparticle, suggesting that the corona may form independently of the surface properties of the nanoparticles, or that no particular property of the NP could dictate formation of the corona. Nonetheless, the authors observed that formation of the corona could affectively mask the identity of the nanoparticle, and this is in agreement with other publications. The transferrin receptor is overexpressed by tumor cells due to their excessive metabolic requirements, and can be used to target drug delivery to the tumor on the basis of transferrin expression.[42] Salvati *et al.* found that when transferrin was conjugated to silica nanoparticles in an attempt to mediate specific uptake by transferrin receptor expressing A549 lung cell line, the level of uptake was not diminished even when transferrin receptor expression by the A549 tumor cell line had been silenced. Upon further investigation, the authors observed that following exposure to biological media containing serum, the ability to target uptake of the particles through the transferrin receptor was lost due to masking of the conjugated transferrin molecules by the protein corona.[43] Thus, the protein corona may limit the ability of the nanoparticle to target specific populations, thereby allowing accumulation in non-desired cell types or tissues. Lastly, the protein corona may differentially affect uptake by the immune system. Using bovine serum albumin (BSA) as a model serum protein to examine the effects of the protein corona, a study by Caruso and

colleagues reported that the adsorption of BSA onto nanoparticles composed of poly(methacrylic acid) (PMA) inhibited uptake in a monocytic cell line, but promoted uptake in a macrophage cell line through the scavenger receptor-A mediated phagocytosis.[44] To delay the uptake of opsonized nanoparticles by macrophages, Rodriguez *et al.* conjugated human CD47 (hCD47) to the polystyrene nanoparticle. CD47 is a glycoprotein found on all mammalian cells that signals through the SIRPα receptor on macrophages to identify the cell as 'self', thereby preventing phagocytic clearance.[45,46] Conjugation of hCD47 or a peptide derivative of hCD47 to the nanoparticle significantly delayed clearance and enhanced circulation when administered to humanized mice, and treatment of the mice with an anti-CD47 antibody reversed this effect to restore clearance of the nanoparticle.[27] This strategy may offer an attractive approach to minimize the clearance of nanoparticles used in diagnostic imaging or other drug delivery applications, and it will be interesting to see how this strategy may be incorporated into nanovaccines for immunotherapy. Thus while few studies have examined the effect of protein adsorption on nanoparticle delivery *in vivo*, it is becoming increasingly apparent that the protein corona is a factor that must be taken into consideration by the investigator when designing a nanovaccine to optimize recognition and uptake of the nanoparticle by the appropriate cell type.

NANOPARTICLE ADJUVANTS AND DC MATURATION

A common goal in the design of vaccine strategies is how to elicit a long-lasting immune response that sufficiently induces the development of effector and memory T cells. The generation of an effector T cell response requires the presentation of cognate peptide/MHC molecules to the T cell by a mature DC bearing T cell costimulatory receptor ligands, such as CD80 (B7-1) and CD86 (B7-2) (Figure 1). DC maturation occurs upon activation, and this can be induced by a number of external and/or internal danger signals such as the presence of TLR agonists, inflammatory cytokines and T cell-derived costimulation.[47] Although some NP platforms may inherently posses some adjuvant-like properties, many strategies have attempted to incorporate some form of DC activation/maturation signal to enhance the immunogenicity of the NP vaccine. In one study, the incorporation of Poly I:C (a TLR3 ligand) and resiquimod into OVA-PLG NPs significantly enhanced the activation, proliferation, and effector function of OVA-specific CTLs when the nanoparticles were targeted to DCs via the DEC205 receptor *in vivo*.[48] The CD40/CD154 costimulatory pathway is a potent activator of innate immune function and has been shown to be critical for the induction of adaptive immune responses in the absence of TLR signals. CD40 is promiscuously expressed throughout the hematopoietic compartment, while its ligand CD154 is primarily expressed by $CD4^+$ T cells following TCR stimulation. Ligation of the CD40 receptor by CD154 or an agonist mAb has been shown to exert potent phenotypic changes in DCs, leading to the enhanced expression of CD80 and CD86, the upregulation of MHC molecules, and production of the pro-inflammatory cytokine, IL-12.[49] Although the administration of an agonist anti-CD40 mAb can lead to severe side effects due to the promiscuous expression of CD40, one study reported that adsorption of an agonist anti-CD40 mAb to a nanoparticle composed of poly(γ-glutamic acid) minimized the systemic effects of anti-CD40 and synergized with the NP to enhance the stimulatory effect of anti-CD40 treatment.[50] Another study that used porous silicon nanoparticles expressing avidin,

found that the potency of a biotinylated agonistic anti-CD40 mAb (FGK) was increased over 30-fold when conjugated to the nanoparticles. This platform led to the enhanced delivery of the nanoparticles to DCs, and significantly promoted the activation and proliferation of B cells when compared to the effect of soluble FGK treatment.[51] Alternatively, the local release of DC maturation signals, such as GM-CSF, can also be used to enhance the adjuvanticity of nanovaccines. Chou, *et al.* reported that the incorporation of GM-CSF and hepatitis B virus surface antigen into a copolymer hydrogel led to the recruitment and activation of DCs that were able to stimulate potent humoral and cell-mediated responses,[52] and a similar approach has been used to eliminate solid tumors in preclinical models using PLG nanoparticle scaffolds that encapsulate GM-CSF while immobilizing tumor lysate and CpG oligonucleotides on its surface.[53] The pulsed release of GM-CSF from the PLG scaffold led to the sustained recruitment, activation and trafficking of DCs bearing antigen from the scaffold to the draining lymph node, leading to tumor immunity that significantly delayed tumor growth and cured over 20% of the tumor-bearing recipients.[53]

The NP platform chosen for vaccine delivery can also possess inherent immunostimulatory properties. Since their initial description as adjuvants 4 decades ago,[54] liposomes have been extensively studied for their versatility in vaccine delivery.[55] Depending on the type of lipid used to fabricate the NP, liposomes can significantly enhance the immunogenicity of the associated antigen, and in some cases are sufficient enough to elicit immune activation on their own. This appears to be particularly true of cationic liposomes which can promote DC activation, CD80/CD86 expression, and chemokine release.[56] Cationic adjuvant formulations (CAF01) are a class of liposomal adjuvants composed of trehalose 6,6′-dibehenate (TDB), a synthetic analogue of a mycobacterial glycolipid antigen, and dimethyldioctadecylammonium (DDA), a lipid with positively charged head groups.[57] CAF01 has been reported to induce high levels of humoral and cellular immune activation when administered with antigen,[58] and this response could be further modified when TDB/DDA liposomes were complexed with poly(I:C) and adsorbed OVA protein, leading to the induction of a long-term, multifunctional memory $CD8^+$ T cell response to OVA.[59] The adjuvanticity of the liposome formulation may also be affected by the structure of the liposome. While thiol conjugation of antigen to unilamellar vesicles induced high titers of antibody production,[60] multilamellar vesicles (MLVs) induced both high antibody titers and T_H1 responses when compared to unilamellar vesicles.[61] Moon *et al.* reported enhanced adjuvanticity of MLVs by embedding Monophosphoryl lipid A, an immunomodulator and TLR4 agonist that is derived from the cell wall of gram-negative bacteria, into the walls of interbilayer-crosslinked multilamellar vesicles (ICMVs).[62,63] Another NP formulation that has been demonstrated to be innately immunogenic are NPs composed of α-Al_2O_3, an alum derivative. When α-Al_2O_3 NPs were conjugated with OVA protein (α-Al_2O_3-OVA) and administered subcutaneously to B16-OVA tumor bearing mice, these recipients rejected the tumors while mice that were immunized with alum and OVA succumbed from tumor burden.[64] Lastly, 25 nm antigen-conjugated Pluronic-stabilized PPS particles were reported to induce DC activation and expression of CD80, CD86, and CD40 through activation of the alternative complement cascade.[65] While various synthetic molecules can be used as adjuvants to direct DC activation, the use of adjuvants that elicit multiple pathways of immune activation (such as complement cascade activation and lymph node remodeling by

mast cell granules[66]) may represent a more promising alternative to synthetic molecular adjuvants by recapitulating additional mechanisms that are involved in the generation of immunity to natural pathogens.

Virus-Like Particles (VLPs)

The issue of biocompatibility can often be addressed by biologically derived, rather than synthetic, nanoparticles. VLPs are one of the most successful examples of such a platform for antigenic display. VLPs are comprised of viral capsid proteins and have the intrinsic ability to self-assemble into particles that range in size between 20 and 100 nm, reflecting the diversity of insect, plant, mammalian, or bacteriophage viruses from which they are derived. Although they resemble live virus in their particulate nature and multivalent structure, VLPs lack a viral genome, making them noninfectious. The small size and repetitive, multivalent structure of VLPs are examples of how size and surface geometry can affect the uptake of NPs by immune cells. Because VLPs are antigenically indistinguishable from infectious virus, they are highly immunogenic, and are able to elicit strong cellular and humoral immune responses, particularly through their interactions with APCs and B cells. Due to their inherent ability to provoke or enhance immune responses, VLPs are often used as vaccines against the virus from which they are derived, (notable examples of this are the FDA-approved human papilloma virus (HPV) and hepatitis B virus (HBV) vaccines) or as platforms to target heterologous molecules that are poorly immunogenic, such as self-antigens, lipids and polysaccharides, small molecules, and non-exposed antigens. Thus, peptides displayed on the surface of a VLP have the potential to present both B- and T-cell epitopes, eliciting a comprehensive humoral and cellular immune response. There are approximately 30 VLP-based therapies undergoing clinical trials today that target a wide range of medical conditions, including both autoimmune and infectious disease, cancer, and addiction.[67,68]

Common methods for attaching antigen to the particle surface include simple chemical conjugation and genetic insertion (Figure 2). Genetic insertion involves site-directed mutagenesis to incorporate a foreign amino acid sequence into the viral coat protein to create multivalent surface presentation of peptides. The limiting factors determining whether a site is amenable to genetic insertion are 1) the inserted peptide must not interfere with protein folding and VLP assembly, and 2) the inserted peptide must be displayed on the outside surface of the VLP. Assembly is further limited by the size of peptide chosen for insertion - insertions of less than 20–30 amino acids are typically well tolerated. Although plant, bacteriophage, and animal viruses such as papillomavirus, rhinovirus, and parvovirus have all been used for genetic display, the most well-characterized are HBV VLPs.[69,70] HBV core antigen (HBcAg) is well suited for genetic insertion of foreign epitopes into a site in the protein's immunodominant loop, or at the N- and C- termini of the protein. Peptides as large as 55 amino acids have been successfully inserted into particles, and the resultant recombinant HBcAg VLPs have been shown to induce strong cellular and humoral immune responses against a variety of targets.[70,71] Two recombinant HBcAg-based vaccines have been tested in humans: the influenza vaccine ACAM-FLU-A,[72] and the malaria vaccine Malariavax (ICC-1132), which displays sequences from the circumsporozoite protein from *Plasmodium falciparum*.[73]

Common methods of chemical conjugation include using bi-functional chemical cross-linkers such as ECDI and SMPH, or creating biotin-streptavidin bridges. As with genetic insertion, the efficiency of chemical conjugation is dependent to a degree on the size of the target antigen. One advantage of targeting peptide epitopes is that they allow for precise targeting of critical epitopes involved in infection or other aspects of the disease pathogenesis. Smaller peptides can also be attached at a very high density to the NP surface, as steric hindrance from secondary and tertiary structures is not a consideration. However, chemical conjugation also opens the door for coupling larger targets, including whole proteins or non-protein molecules, to the NP surface. These conjugates have the potential to induce a broad range of antibodies capable of recognizing both linear and conformational epitopes on the target molecule. For example, as many as 240 molecules of a 12-amino acid peptide can be linked to VLPs composed of the RNA bacteriophage Qβ,[74,75] while only approximately 18 copies of a 34 kDa IL-17 homodimer can be linked to Qβ VLPs.[76] Regardless, the Qβ VLP platform has been widely used for the development of VLP-based vaccines currently in human clinical trials, and many more are in development. These include vaccines targeting amyloid-β,[74,77] angiotensin II[78,79] and nicotine,[80,81] for treatment of Alzheimer's disease, hypertension, and nicotine dependence, respectively.

Filamentous Nanostructures

In addition to serving as natural NP platforms, VLPs have also helped inform the development of synthetic NPs. For example, the filamentous shape of several viruses led to the development of likewise filamentous nanostructures including nanorods, nanowires and nanotubes, which are gaining momentum for their application in the fields of molecular imaging[82], biomarker detection[83], drug and gene delivery[84], tissue engineering[85] and radiation therapies,[86] among others. While much of this work is beyond the scope of this review, we can highlight some observations with regards to nanorod and carbon nanotube (CNT) interactions with the immune system. A recent study showed that displaying the major protective antigen, fusion (F) protein, of respiratory syncytial virus (RSV) on the surface of gold nanorods induced potent human T cell responses when co-cultured with DCs treated with the vaccine. Notably, the vaccine not only took into consideration the importance of the NP core's filamentous shape in securing its immunogenicity, but also the conformation of the F protein epitopes on its surface. Successful conjugation of the full-length antigenic protein and therefore maintenance of its secondary structure was likely essential in mimicking free protein and delivering protective viral antigens to human APCs.[87] While this study highlights the importance of NP shape on the immune response, it also calls attention to a common deficit in CNT research for biomedical application: much of the research is conducted *in vitro* and, accordingly, there is a limited amount of *in vivo* data on CNT immunotoxicity. A more comprehensive review on the topic has been published by Andersen, *et al.* [88] As with nanoparticle composition in general, the wide array of CNT formulations makes it difficult to compare results across experiments. For example, Sanjiv S Gambhir, *et al*. used cyclic RGD peptide-conjugated single-walled CNTs as photoacoustic molecular imaging agents in tumor-bearing nude mice. While the experiments were performed using live mice, no toxicity studies were performed, and the mice were sacrificed within 4 hours of the intravenous injection of particles[82]. The same group recently showed that undecorated gold nanorods could also be used to image subcutaneous

xenografts of ovarian cancer cell tumors in nude mice. Again, no immune assays were performed, though the imaging was carried out up to 2 days following injection[89]. A more immunology-focused *in vitro* study by Son, *et al*. showed that nanorods functionalized with immune cell ligands such as mannose and RGD peptides formed nanobridges that could increase immune cell proximity, thereby facilitating antigen presentation and cytokine release. When co-cultured with dendritic cells and T cells, longer (4μm as compared to 1–2 μm) nanorods enhanced pro-inflammatory IL-2 and IFN-γ secretion. This work, although strictly *in vitro*, provides an intriguing alternative to direct delivery of antigens and adjuvants to APCs as a means of modulating immune responses[90]. In contrast, Aldinucci *et al*. recently reported that multi-walled CNT scaffolds incorporated into DC cultures resulted in a lower immunogenic profile (transcripts for proinflammatory cytokines such as IL-12, IL-23 were undetectable) and did not induce cell death[85]. Other examples of synthetic nanoparticles that have been used as vaccine carriers include co-polymer hydrogels or 'nanogels', cationic liposomes (discussed previously) and biodegradable poly(lactide-*co*-glycolide) (PLGA or PLG) nanoparticles.

NANOPARTICLES AND T CELL ACTIVATION

Although current vaccines are able to elicit potent humoral immunity, the ability to induce a long-lasting, protective memory $CD8^+$ T cell response remains a significant challenge in the design of rational vaccines. Chronic viral infections such as HIV that may persist in cellular reservoirs during antiviral therapy, and proliferating tumor cells that are resistant to chemotherapy necessitate elimination by cytotoxic T cells. While intracellular antigens and viral proteins present in the cytosol are subsequently degraded by the proteasome and directed for presentation in the context of MHC class I to $CD8^+$ T cells, antigens that are acquired exogenously by DCs typically do not access the cytosol, but are degraded in the endo-lysosome and enter the MHC class II loading pathway.[91] This presents a significant barrier to the generation of cytotoxic $CD8^+$ T cell responses to soluble or endocytosed antigens, since the ability to cross-present exogenously acquired antigen to $CD8^+$ T cells is limited to specialized populations of DCs, such as the population of splenic and lymph node resident $CD8\alpha^+DEC205^+$ DCs and the mucosal population of $CD103^+$ expressing DCs.[92] Indeed, the priming of $CD4^+$ and $CD8^+$ T cells may be preferentially mediated by divergent subsets of DCs *in vivo*.[93] To overcome this obstacle, one PLG nanoparticle vaccine strategy incorporated the use of the antibodies targeting the DEC205 receptor that is expressed by $CD8\alpha^+$ DCs to promote cross-presentation of the vaccine to $CD8^+$ T cells.[48] However, a study that immobilized anti-DEC205 antibodies to the surface of OVA-bearing PLG particles by avidin-conjugation reported that crosslinking of the DEC205 receptor led to increased production of IL-10 by DCs and T cells, even when the nanoparticle was used to boost immunity induced by an OVA-CFA immunization.[94] This observation is not entirely surprising since a number of studies exist that have targeted chimeric antibody-antigen complexes to DEC205 for the purpose of immunoregulation.[95–98] The discrepancy between the two nanovaccine studies may be attributed to the incorporation of TLR ligands with regards to the former study,[48] and a high density of anti-DEC205 antibody in the latter study,[94] which purportedly led to increased DEC205 cross-linking.

Other strategies that have been used to enhance the cross-presentation of nanovaccines to $CD8^+$ T cells involve optimizing parameters of the NP design such as size, material and charge. In agreement with the work published by Hubbell and colleagues,[65] Li *et al.* observed that smaller (60 nm) α-Al_2O_3-OVA NPs showed better accumulation in the lymph nodes draining the site of injection and induced more robust OT-I proliferation than larger (200 nm) α-Al_2O_3-OVA NPs.[64] Interestingly however, 25 nm α-Fe_2O_3-OVA NP did not induce similar levels of OT-I proliferation, suggesting that the ability of α-Al_2O_3-OVA NP to induce optimal cross-presentation was a consequence of the inherent functional properties of α-Al_2O_3 NP. Li and colleagues observed that the superior potential of the α-Al_2O_3-OVA NP to cross-prime $CD8^+$ T cells could be attributed to the ability of the α-Al_2O_3-OVA NPs to access the autophagosome, a subcellular compartment that degrades damaged or decaying organelles and is consequently rich with endogenous antigens that can be presented via the MHC class I pathway.[99] To this extent, the authors found that cross-presentation of α-Al_2O_3-OVA could be inhibited by treatment of α-Al_2O_3-OVA-bearing DCs with Brefeldin A, an inhibitor the non-canonical autophagy pathway.[100] Cationic nanoparticles bearing protein antigens have also been reported to promote cross-priming of $CD8^+$ T cells due to their ability to rupture the endo-lysosome and release its contents into the cytosol where the antigens can be directed towards the MHC class I pathway. OVA protein adsorbed onto cationic DDA/TDB NPs incorporating poly(I:C) were demonstrated to generate long-term central and effector memory $CD8^+$ T cell responses to SIINFEKL, an MHC class I-restricted peptide derived from OVA, and that this response was further enhanced if the DDA/TDB-OVA NPs were fabricated by the double emulsion method to form MLVs.[59] The observation that MLVs were more efficient at cross-priming a $CD8^+$ T cell response is in agreement with the study by Moon *et al.* in which MLVs and ICMVs induced a greater $CD8^+$ T cell response to OVA than did monolayer liposomes encapsulating OVA.[62] MLVs may better promote cross-presentation due to their stability in the extracellular environment, persistence in the lymph nodes, and prolonged antigen delivery. Reduction-sensitive antigen-conjugation to NPs has been reported to better facilitate the cross-priming of cytotoxic T cells when compared to antigen delivery by encapsulation or reduction-insensitive conjugation,[101] and this has been suggested to be the result of conjugated antigens rapidly accessing the endosome in their native form.

Artificial Antigen Presenting Cells

One strategy that obviates the necessity of targeting nanoparticle vaccines to the appropriate APC population for the development of protective immunity is the use of artificial APCs (aAPCs). This approach involves the use of nanoparticles that express TCR agonists, with or without antibodies that stimulate costimulatory receptors to directly activate T cells. In one study, 60-160 $OVA_{323-339}$/I-A^d molecules were incorporated into 60 nm liposomes through biotin-avidin conjugation, leading to the production of IL-2 by $OVA_{323-339}$ peptide-specific $CD4^+$ T cell hybridomas.[102] Whether or not this approach led to the induction of a functional T cell response was not investigated, although the likely outcome of TCR stimulation in the absence of costimulation is anergy.[103] In another study, aAPCs were manufactured by coupling HLA-Ig and anti-CD28 antibodies to the surface of polystyrene particles pulsed with viral antigen. The culture of $CD8^+$ T cells with these aAPCs promoted the generation and expansion of both low and high-affinity virus-specific CTLs that were

capable of mediating cytotoxicity against cells endogenously presenting CMV and MART-1 viral antigens.[104] Interestingly, one study reported that the administration of aminated iron-oxide nanoparticles coated with peptide/MHC complexes bearing diabetogenic epitopes from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), a pancreatic β cell antigen, induced the expansion of low-avidity IGRP reactive $CD8^+$ T cells *in vivo*.[105] However, rather than enhancing autoimmunity diabetes, this treatment led to the induction of a regulatory $CD8^+$ T cell population that suppressed the development of type 1 diabetes in the NOD mouse. This strategy was also effective in preventing diabetes onset and restoring euglycemia in a humanized NOD model of diabetes if IGRP and Insulin-HLA complexes were conjugated to the nanoparticle surface. The ability of this platform to induce tolerance was dependent upon perforin-mediated killing of APCs, and the induction of *indoleamine*-2,3-*dioxygenase* (IDO) since treatment with 1-methyl-[D]-tryptophan, an IDO inhibitor, prevented the therapeutic effect of the nanoparticle administration.[105] Since this study did not incorporate costimulatory agonists into the nanoparticle platform, it remains to be determined whether or not the administration of aAPCs can also be effective at boosting anti-tumor or anti-viral responses *in vivo*.

Nanoparticles and Tolerance

The induction of antigen-specific immunological tolerance has vast potential for the treatment of autoimmunity, allergy, and transplant rejection. Central to each of these disorders is the dysregulation of adaptive immunity caused by the breakdown of peripheral tolerance,[106] and the subsequent induction of T cell-mediated responses that target tissue and environmental antigens. Treatment of these disorders are often limited to broad immunosuppression, and as such, the induction of immunological tolerance has occupied an area of intense interest for the better part of a century and remains the holy grail of basic and translational scientific research for the regulation of immune-mediated diseases.[107] While nanoparticles as platforms for immunotherapy have most often been described in the context of vaccination delivery, the immunomodulatory potential of nanoparticles has warranted their investigation as a tool for immunoregulation. To this extent, strategies have been developed with the purpose of inhibiting T cell function at the level of the APC-T cell interaction or by targeting pathogenic T cells directly and indirectly via the activation of Tregs.[108] Moreover, many of the same principles critical for promoting T cell activation in vaccination strategies are important for directing approaches for the induction of T cell tolerance including the route of administration, properties of the nanoparticle platform – size and surface charge, and the use of adjuvants are all parameters that can dictate the fate of a T cell during antigen-recognition.

The intravenous route of administration has long been associated with the induction of immunological unresponsiveness. The Sulzberger-Chase studies dating back to the late 1920's reported that the intravenous administration of haptens led to the induction of hapten-specific, immunological unresponsiveness,[109] and intravenous soluble protein or peptide therapy has also been a potent strategy for inducing peripheral tolerance in experimental settings.[110] Particulate substances injected intravenously are rapidly engulfed and degraded by specialized phagocytes in the spleen and liver, limiting the duration of antigen exposure which is critical for mounting an effective immune response. Furthermore, marginal zone

macrophages (MZ MΦ) of the spleen clear the body of apoptotic cells and debris, while concomitantly suppressing adaptive immune responses to the re-presented self-antigens.[111] This second point is exemplified by the observation that disruption of the MZ MΦs by treatment with low-dose clodronate liposomes has been observed to enhance the development of autoimmune systemic lupus erythematosus in genetically susceptible mice.[111] Although the exact mechanism behind the unresponsiveness induced by the intravenous administration of antigen is not known, it may be due in part to the short availability of antigens presented intravenously,[112] and the immature or tolerogenic phenotype of the APCs that encounter, acquire, and present the antigens to T cells.

One approach that was born out of the Sulzberger-Chase phenomenon was a form of coupled cell tolerance using ECDI-fixed, antigen-coupled syngeneic splenocytes (Ag-SP).[113] Ag-SP is a powerful strategy for the induction of peripheral immunological tolerance, and has been demonstrated to both prevent and treat aberrant T cell responses in experimental models of autoimmunity, allergy, and transplant rejection.[107,113,114] The use of ECDI chemistry to conjugate antigens to the surface of splenocytes also causes rapid apoptosis in the fixed cells following intravenous infusion,[115,116] thereby leading to their subsequent uptake by MZ MΦs via scavenger receptors.[117] The downstream effect of Ag-SP internalization is the induction of a regulatory phenotype by the MZ MΦ; these cells upregulate the production of immunosuppressive cytokines IL-10 and TGF-β, exhibit increased expression of the regulatory costimulatory ligand PD-L1, and fail to enhance the expression of positive costimulatory ligands such as CD80 and CD86.[117] In the absence of positive costimulation, these inhibitory signals converge upon a T cell during antigen-recognition to constrain the production of IL-2, culminating in a state of T cell anergy.[103] In addition, antigen presentation in the presence of IL-10 and TGF-β is a potent immunoregulatory milieu leading to the activation of antigen-specific Tregs which synergizes with anergy to mediate Ag-SP tolerance.[114,117] This approach can be used to selectively inhibit responses to either antigenic peptides or whole proteins, and has significant potential for limiting the progression of autoimmune disease by epitope spreading.[118]

We have recently demonstrated that the intravenous infusion of antigen-coupled, highly carboxylated 500 nm polystyrene and PLG microparticles can function effectively as surrogates for the Ag-SP to promote the establishment of peripheral T cell tolerance (Figure 3).[118] While biodegradable PLG NPs are well-characterized and frequent candidates for encapsulated antigen delivery, studies showing their applicability as antigen platforms were limited. Tolerance induced by ECDI-fixed, antigen-coupled microparticles (Ag-MP) led to a defect in in the proliferation of antigen-specific T cells, both *in vitro* and *in vivo*; additionally, proliferation could be rescued *in vitro* if exogenous IL-2 was present during antigen-specific restimulation, and these observations are consistent with the induction of an anergic state.[119] T cell differentiation and cytokine production was also largely inhibited by Ag-MP treatment, since antigen-specific T cells isolated from mice that were challenged with Ag-MP failed to produce IFN-γ and IL-17 when they were restimulated *ex vivo* with specific antigen or by PMA and ionomycin. Tolerance induction mediated by treatment with Ag-MP was dependent upon the route of administration, since intravenous injection of Ag-

MP led to the establishment of tolerance while subcutaneous administration did not. Size was also a determining factor in the induction of tolerance by Ag-MP in that 500 nm Ag-MP were efficient at inducing tolerance for the prevention of experimental autoimmune encephalomyelitis (EAE), a mouse model of the Th1/17 autoimmune diseases multiple sclerosis, while 100 nm Ag-MP failed to induce tolerance and protect mice from developing disease and particles larger than 1 μm were not as efficient. Ag-MPs were found in the spleen, liver, and lungs of mice following intravenous infusion, and analyses of the spleen via immunohistochemistry at 3 hours post-injection showed localization of the fluorescent Ag-MP with the macrophage scavenger receptors MARCO and SIGNR-1, suggesting a possible role for scavenger receptor mediated endocytosis of the Ag-MP. To this extent, mice genetically deficient in MARCO could not be rendered tolerant by Ag-MP but were still susceptible to tolerance induction by Ag-SP, demonstrating a non-redundant role for MARCO in the uptake of Ag-MP. MARCO has been shown to play a role in the clearance of polyanionic substances such as LPS, and the crosslinking of antigen onto the carboxylated particles may provide repeated motifs that solicit engagement by MARCO. In turn, MARCO-mediated endocytosis has been reported to affect innate immune deactivation and tolerance[120].

The incorporation of tolerogenic ligands into nanoparticle platforms has also been described. The cationic polymer polyamine polyethelenimine (PEI) can be used to form conjugates with DNA (DNA/PEI) as a delivery platform for plasmid DNA transduction. However, due to the presence of CpG oligonucleotides present in DNA this approach has been demonstrated to promote significant immune activation and pro-inflammatory cytokine production.[121,122] Interestingly, a recent study by Huang *et al.* reported that DNA/PEI nanoparticles could promote the induction of tolerance *in vivo*. When modified DNA/PEI nanoparticles lacking CpG motifs were administered to mice with rheumatoid arthritis, a significant therapeutic effect was observed. Treatment with DNA/PEI inhibited joint inflammation, suppressed antigen-specific T cells responses, and led to the induction of Tregs through IFN-αβ-mediated IDO expression by DCs.[123] Alternatively, activation of the aryl hydrocarbon receptor (AHR) by the ligand 2-(1′*H* -indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) promotes a tolerogenic phenotype in DCs that leads to the differentiation of Tregs.[124] A recent study reported that the conjugation of CNS myelin peptides and ITE to 60 nm gold particles (NP-ITE) was found to suppress the development of EAE, and therapeutically ameliorated ongoing EAE disease following repeated administration.[125] Furthermore, the transfer of $CD4^+$ T cells from NP-ITE treated mice suppressed the induction of EAE in naïve recipients, but only if Tregs were present in the transferred $CD4^+$ T cell population since depletion of $FoxP3^+$ cells expressing a GFP reporter abrogated the suppressive effect of the transferred $CD4^+$ T cells.[125] Together, these data illustrate the usefulness of incorporating tolerogenic ligands into nanoparticles as adjuvants to promote regulatory outcomes.

CONCLUSIONS

The use of antigen-conjugated nanoparticles for vaccine delivery is a rapidly expanding method of immunotherapy. For the investigator, a significant level of attention must be invested in the design of the nanoparticle platform as the nanoparticle material, size, shape,

charge, route, and the incorporation of ligands can be manipulated to affect nanoparticle interaction with the immune system to achieve the desired outcome. Evidence from recent publications that have reported the use of nanoparticles for the induction of immunological tolerance has suggested that these same parameters can be used to effectively target immunoregulatory pathways for the treatment of aberrant T cell responses and experimental models of autoimmunity. While the use of antigen-conjugated nanoparticles appears promising as an immunotherapy under experimental settings, its viability as a clinical application remains to be determined. Future studies will need to examine dosing and toxicology associated with the infusion antigen-conjugated nanoparticles in preclinical models in order for the methodology to progress to the clinic.

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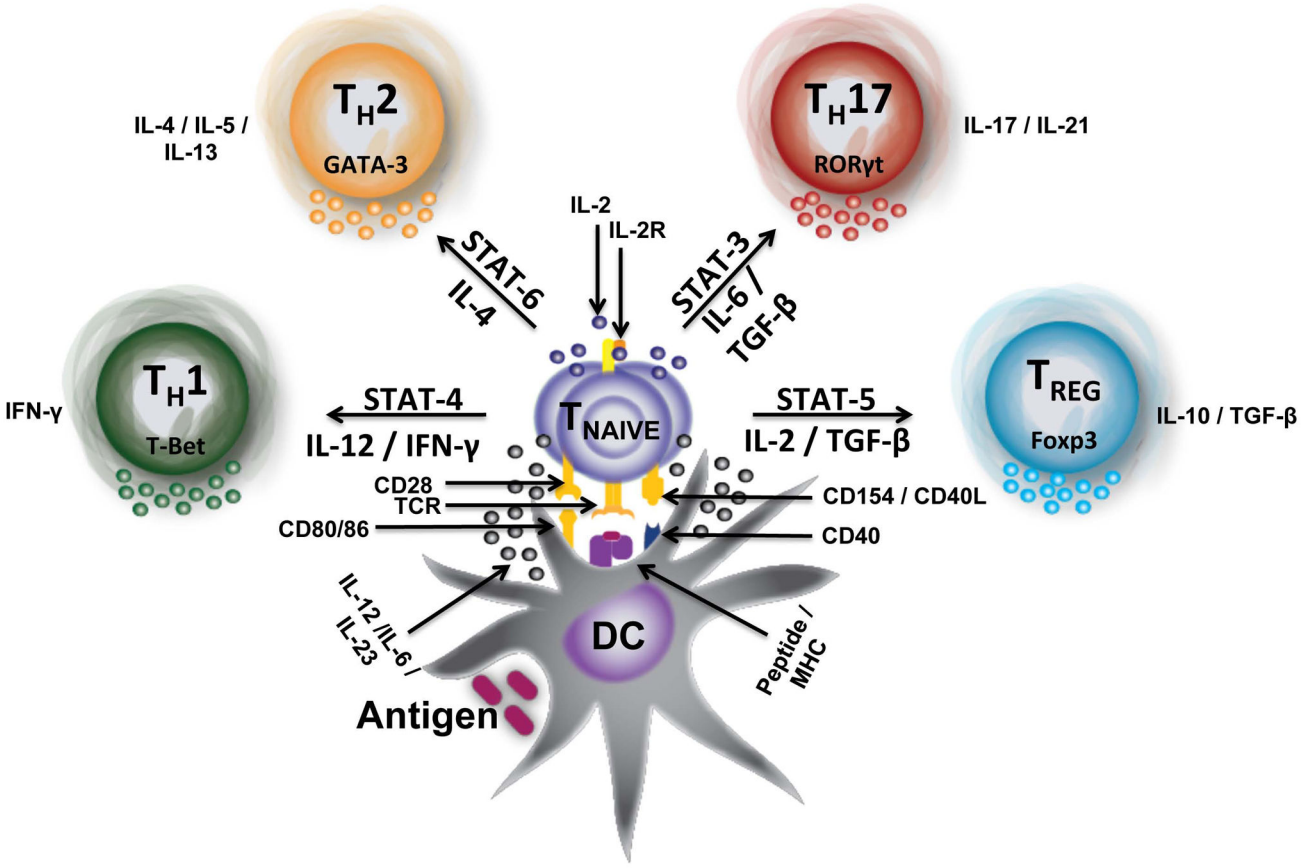

FIGURE 1.

Dendritic cells initiate T cell immunity in the lymph nodes. A mature DC encounters antigen in the lymph nodes where it processes and cross-presents the antigen in the context of peptide/MHC molecules to T cells. A T cell bearing a receptor (TCR) of cognate specificity to the presented peptide/MHC is induced by TCR stimulation (signal 1) to express CD40L, leading to activation of the DC through CD40 engagement. DC activation results in increased costimulation to the T cell through the B7/C28 pathway (signal 2). TCR stimulation in the presence of CD28 signaling results in T cell activation, IL-2 dependent proliferation, and differentiation into an effector T cell. The cytokine milieu directs T cell differentiation along one of the helper T cell (T_H) lineages.

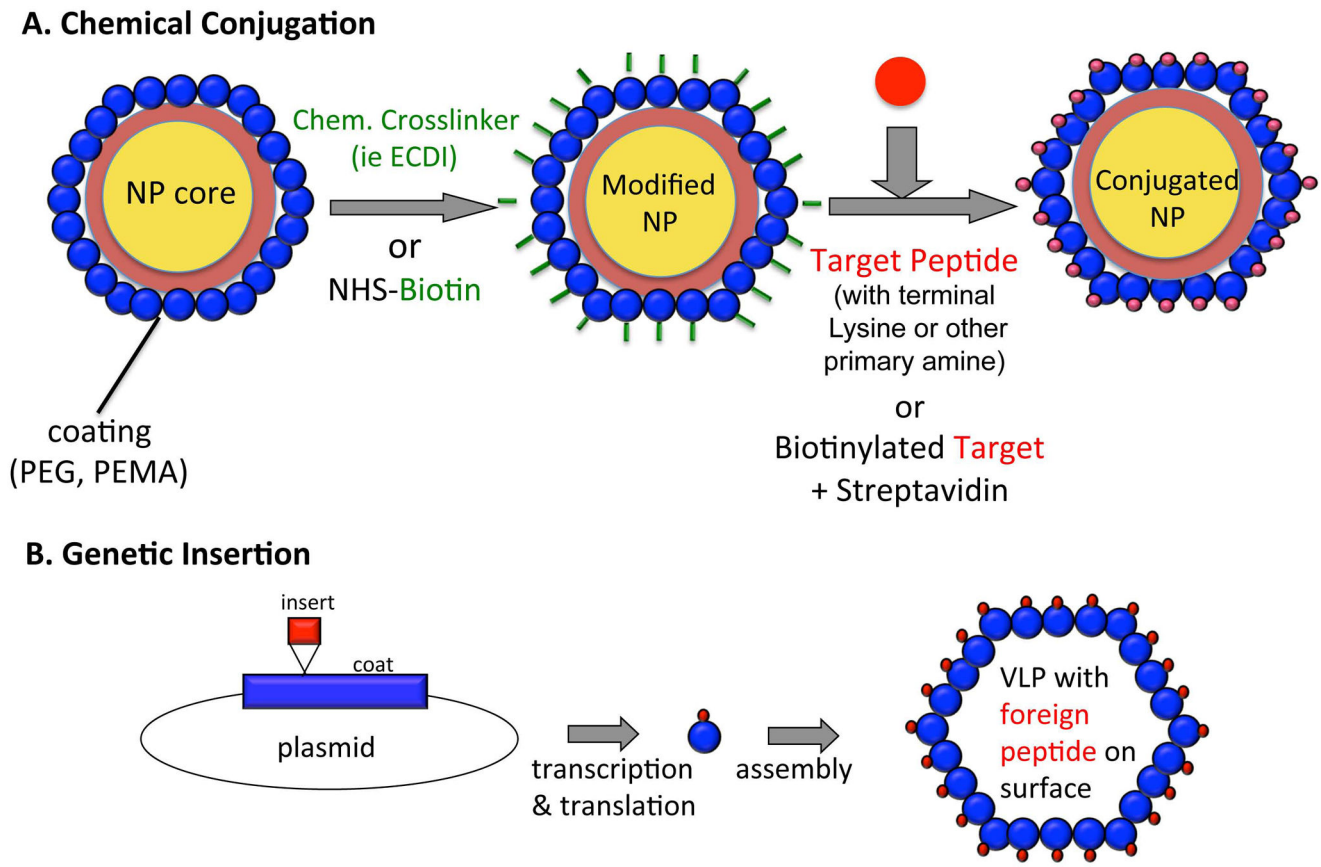

FIGURE 2.

Methods of displaying antigen on the particle surface. A) Chemical conjugation uses chemical crosslinkers or biotin-streptavidin cross-bridges to link the desired peptide to the NP surface. B) Genetic insertion results in recombinant particles in which the peptide of interest is inserted into a coat protein and displayed on the surface of a VLP following successful translation.

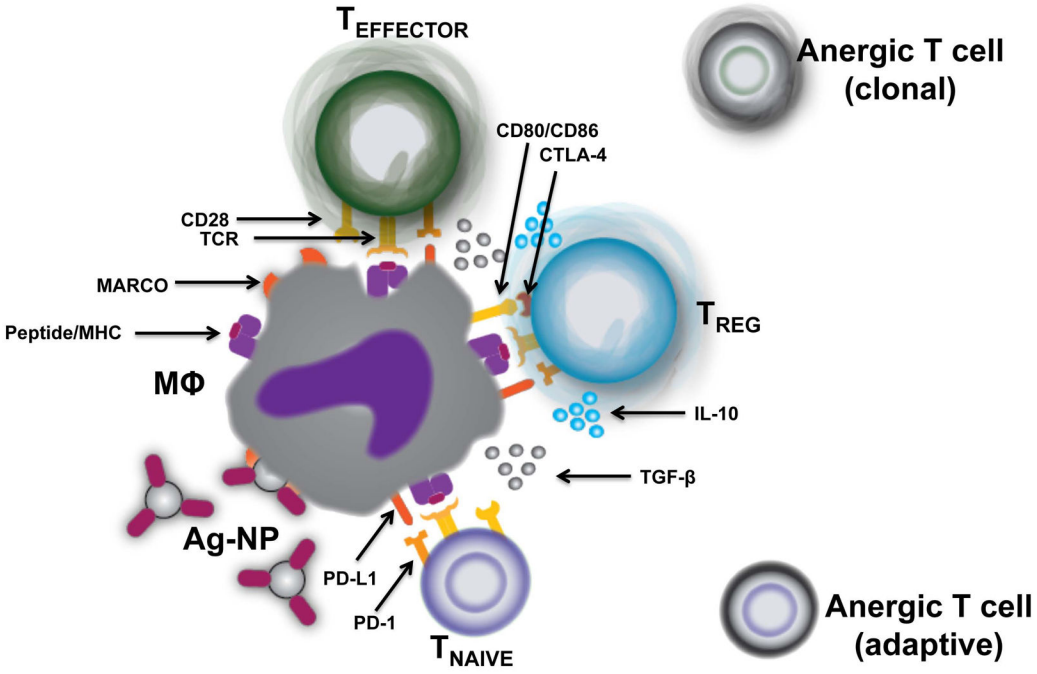

FIGURE 3.

The uptake of Ag-NP by splenic marginal zone macrophages leads to the induction of T cell tolerance. A) Ag-NP in the spleen are phagocytosed by marginal zone macrophages (MΦ) through the scavenger receptor MARCO, leading to a tolerogenic phenotype and an increase in PD-L1 and immunoregulatory cytokine expression. B) The immunoregulatory milieu supports the induction and/or activation of $Foxp3^{+}$ Tregs that may limit the ability of splenic APCs to prime naïve T cells by producing immunosuppressive cytokines and by CTLA-4-mediated trans-endocytosis of CD80/CD86 molecules. Cross-presentation of the antigen to a T cell (signal 1) in the absence of costimulation (signal 2) causes abortive activation and defective IL-2 production, leading to the induction of adaptive or clonal T cell anergy in naïve and differentiated effector T cells, respectively.