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Immunological Principles Guiding the Rational Design of Particles for Vaccine Delivery

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ABSTRACT

Despite the immense public health successes of immunization over the past century, effective vaccines are still lacking for globally important pathogens such as human immunodeficiency virus (HIV), malaria, and tuberculosis (TB). Exciting recent advances in immunology and biotechnology over the past few decades have facilitated a shift from empirical to rational vaccine design, opening possibilities for improved vaccines. Some of the most important advancements include: (i) the purification of subunit antigens with high safety profiles, (ii) the identification of innate pattern recognition receptors (PRRs) and cognate agonists responsible for inducing immune responses, and (iii) developments in nano- and microparticle fabrication and characterization techniques. The latter advances now allow highly tunable physicochemical properties of particle-based vaccines, including composition, size, shape, surface characteristics, and degradability. We propose that enhanced collaborative efforts between immunology and materials science will give rise to next-generation vaccines. This process will be significantly aided by a greater understanding of the immunological principles guiding vaccine antigenicity, immunogenicity, and efficacy. With specific emphasis on PRR-targeted adjuvants and particle physicochemical properties, this review aims to provide an overview of the current literature to guide and focus rational particle-based vaccine design efforts.

KEYWORDS: adjuvant; vaccine particles; codelivery; antigen presentation; pattern recognition receptors; antigen presenting cell; lymph node trafficking; subunit antigen; TLR; NLR

VOCABULARY

Antigen, unique molecule (*e.g.*, protein, peptide, polysaccharide) that is specifically recognized by the adaptive immune system; **adjuvant**, a component (*e.g.*, alum, PRR agonist) or characteristic (*e.g.*, particle-based delivery system) of a vaccine formulation that enhances the quality or quantity of the induced immune response; **pattern recognition receptor (PRR)**, cellular receptors that recognize pathogen- and danger-associated molecular patterns (PAMPs and DAMPs); **toll-like receptor (TLR)**, PRRs on the cell surface membrane (TLR1, TLR2, TLR4, TLR5, TLR6) that recognize bacterial products such as lipopolysaccharide, lipoteichoic acids, lipoproteins, and flagellum and on the endosomal membrane (TLR3, TLR7, TLR8, TLR9) that recognize viral nucleic acids, which can be accessed during viral replication or upon intracellular degradation; **nucleotide-binding oligomerization domain-like receptors (NOD-like receptor, NLR)**, PRRs in the cytoplasm. NLRP3 senses cellular damage and stress. NOD receptors recognize bacterial peptidoglycan (PGN); **agonist**, a molecule that specifically interacts with a cellular receptor (*e.g.*, PRR) to activate a physiological response, such as an immune response; **endocytosis**, active cellular internalization that can occur *via* a variety of cell surface receptors, such as PRRs.

Since the introduction of the smallpox vaccine by Edward Jenner in 1798, vaccines have been created to protect against a range of infectious diseases.[1] The eradication of smallpox was announced by the World Health Organization (WHO) in 1979, poliovirus is now nearing global eradication, and measles is controlled in most parts of the world. With the exception of safe water, vaccination is considered the most effective health intervention ever developed.[2] Despite successes to date, safe and efficacious vaccines are still lacking for many important chronic human pathogens, such as malaria, tuberculosis (TB), and human immunodeficiency virus (HIV).

Most current vaccines are derived from either live-attenuated or inactivated pathogens or toxins (*i.e.*, toxoid). Live-attenuated vaccines contain pathogens that have been weakened through selective propagation (*i.e.*, multiple passages in non-human hosts) to reduce their replicative fitness and prevent onward transmission. Administration of these vaccines typically results in mild to asymptomatic infection, but generates long-lived immunity similar to that observed in individuals who recover from natural infection. However, live-attenuated vaccines have the potential to cause disease, especially in individuals with compromised immune systems. Inactivated and toxoid vaccines contain pathogens or toxins, respectively, that are inactivated by heat or chemical (*e.g.*, formaldehyde) treatment. Inactivated and toxoid vaccines are potentially safer than live-attenuated vaccines, but material derived from pathogens inherently contains microbial components that can increase the risks of unwanted side effects, such as excessive inflammation. Batch-to-batch variation and pathogens with difficult or problematic culturing protocols are additional disadvantages associated with live-attenuated, inactivated, and toxoid vaccines. Enabled by advances in bioinformatics (*i.e.*,

immunoinformatics)[3,4] recombinant DNA technologies, and genetic engineering, the development of protein and peptide subunit antigens has opened possibilities for rationally designing safer vaccines for a wider range of applications including cancer and chronic infections.[5-7] However, in their purified, soluble form, protein and peptide antigens are poorly immunogenic; that is, immunization generally does not induce responses that are sufficient to result in protective immunity. This is because: (i) immunostimulating microbial components are not present in these purified antigens, and (ii) diffusion and clearance of soluble material inhibits the required local concentration of antigen necessary for immune response induction. Particulate systems are inherently more immunogenic than soluble systems, thus subunit antigens require particle-based delivery systems and adjuvants to induce immune responses.[6,8]

In addition to subunit antigen-based vaccines, most vaccines require adjuvants to induce sufficient immune responses ("adjuvare" is Latin for "to help").[9] Currently licensed vaccines are formulated with either aluminum salts (*e.g.*, aluminum oxohydroxide, aluminum hydroxyphosphate) (also known as "alum") or oil-in-water emulsions, which act as both particulate vaccine delivery vehicles and immunostimulants.[10,11] Both alum and emulsion adjuvants were empirically identified and the mechanisms of vaccine enhancement remain poorly defined.[12,13] However, these adjuvants boost immune responses and in particular, neutralizing antibodies, which are a correlate of protection for most human pathogens for which there are currently licensed vaccines.[14] For several major pathogens such as malaria, TB, and HIV, effective vaccines have been elusive and traditional approaches of vaccine development have either failed or have been too weakly protective to be widely useful.[15-17] Recent advances in

biotechnology and a greater understanding of the immunological basis for effective vaccination may facilitate the rational design of next-generation vaccines;[18] particularly the identification of immunopotentiating molecules that specifically activate pattern recognition receptors (PRRs) on innate immune cells, which could form the basis of advanced adjuvant formulations,[19] and highly tunable particle-based delivery systems for precise delivery of antigens and adjuvants *in vivo*.[20] This review provides an overview of vaccine immunology as it relates to PRR activation and the effects of vaccine particle physicochemical properties on the quality and magnitude of immune responses to immunization. Two classes of PRRs with significant potential as targets for next-generation adjuvants are highlighted: toll-like receptors (TLRs) and NOD-like receptors (NLRs). Additionally, important recent studies that have elucidated the effects of particle size, shape, surface characteristics, and degradability on the efficacy of particle-based vaccines are discussed in detail. The overarching aim of this review is to contextualize how adjuvant and particle characteristics can be modularly engineered to achieve desired immunization outcomes.

OVERVIEW OF THE GENERATION OF PROTECTIVE IMMUNE RESPONSES BY VACCINATION

Vaccine Administration and Trafficking. The majority of currently utilized vaccines are administered intramuscularly (*i.e.*, direct injection into the skeletal muscle), a route associated with low reactogenicity, which is highly favorable for licensure. Tissue damage at the site of administration triggers local innate immune responses (*e.g.*,

cytokine and chemokine secretion) by muscle cells and muscle-resident immune cells (reviewed in Liang *et al.*[21]). This leads to local inflammation and the infiltration of immune cells from the circulation to the site of injection, particularly neutrophils and antigen presenting cells (APCs) such as monocytes/macrophages and dendritic cells (DCs), a subset of immune cells highly specialized for antigen capture and presentation. DCs, both migratory and those resident within the muscle, efficiently capture antigen from the extracellular environment *via* endocytosis (*e.g.*, phagocytosis, macropinocytosis), which can occur *via* a variety of cell surface receptors,[22-24] including PRRs that recognize pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively) (Figure 1a).[19] Internalization of antigen and the engagement of PRRs induce DC maturation, upregulatation of antigen processing machinery,[25] and presentation of intracellularly degraded antigen fragments on the cell surface by complexation with major histocompatibility complex (MHC) molecules (Figure 1a). In addition, DC maturation drives changes in the expression patterns of surface chemokine receptors (*e.g.*, CCR7), which results in migration out of the muscle to lymphoid organs *via* the blood or lymphatic system.[26] Some vaccine material may also traffic to lymph nodes *via* convective flow from the interstitium without assistance from migratory APCs (Figure 1b).[27]

Priming of Adaptive Immune Responses in Lymph Nodes. Lymph nodes are located in anatomically strategic positions to sample antigens and facilitate adaptive immune responses, which are dependent upon two important subsets of lymphocytes, T cells and B cells (Figure 1c). Within lymphoid tissues, T cells and B cells localize to two functionally partitioned areas termed the T cell zones and B cell zones. Mature DCs

arriving from the tissues enter the T cell zone, where T cell recognition *via* the T cell receptor (TCR) of intracellularly processed antigen presented in the context of MHC drives the activation of antigen-specific naïve T cells (often termed signal I; T cell signaling reviewed in Mantegazza *et al*.[28]). Alternatively, antigens that have entered the lymph nodes without internalization and trafficking by DCs at the injection site may be phagocytosed and processed by sub-capsular sinus (SCS) macrophages.[29,30] If sufficiently small, antigen may also directly diffuse into the T cell zone *via* conduits established by fibroblastic reticular cells,[31,32] where lymph-node resident DCs can internalize and present antigens to T cells.[33] DCs simultaneously express co-stimulatory signals on the cell surface (*i.e*., CD80/CD86) (signal II), and a cocktail of secreted cytokines (signal III) that act in concert to fine tune the activation and differentiation program of responding T cells, thereby tailoring the host immune response to the nature of the pathogen.[34,35] Two common types of T cells have been delineated based upon differing glycoprotein co-receptor components of the TCR, either CD4+ or CD8+ (Figure 1c). DC-mediated activation of CD4+ and CD8+ T cells triggers proliferation and differentiation into immune effectors, which act both directly and indirectly to clear infections and prevent disease. In addition, proliferating T cells have the capacity to differentiate into long-lived populations of cells primed for rapid response to secondary exposure, the immunological memory that is a hallmark of adaptive immunity.

CD8+ T cells recognize antigen peptide fragments (~8-9 amino acids) in the context of MHC class I, which is ubiquitously expressed by every host cell and predominantly used to present antigens localized within the cytoplasm. Endocytosed material can also be presented *via* MHC class I, a process termed “cross-presentation” (Figure 1a). The

cellular mechanisms that enable cross-presentation may include several overlapping pathways (reviewed in Joffre *et al.*[36]). Materials within the endosomes can be degraded into peptide fragments, allowing import into the endoplasmic reticulum (ER) and presentation *via* classical MHC class I pathways.[36] Alternatively, degraded peptides can be imported directly back into phagosomes (vacuolar pathway) for MHC I loading and transport to the cell surface (reviewed in Ma *et al.*[37]). Activated antigen-specific CD8+ T cells (*i.e.*, cytotoxic T lymphocytes, CTLs) leave lymphoid sites and actively seek out and kill infected cells displaying cognate peptides *via* MHC I on the cell surface. This cytotoxic/cytolytic ability is crucial in the maintenance of effective immune control against intracellular pathogens and cancer.[38] CD4+ T cells recognize peptides (9-20 amino acids) complexed with MHC class II molecules, which are mainly expressed by professional APCs (*i.e.*, DCs, macrophages/monocytes, B cells) MHC class II presentation is mainly used for extracellular antigens endocytosed and degraded in endosomal/lysosomal compartments (reviewed in Roche *et al.*[39]). Activated CD4+ T cells, or T helper (Th) cells, provide critical support to many aspects of the immune response, including CTL and serum antibody responses.[40] While numerous specialized subsets of Th cells are recognized in the literature, such as Th1 (IFN-γ-producing), Th2 (IL-4- and IL-5-producing), Th17, and regulatory T cells (Treg), the CD4+ T cell compartment displays incredible plasticity, both in terms of phenotype (*i.e.*, surface marker expression) and function (*i.e.*, cytokine and chemokine secretion) (reviewed in Oestreich *et al.*[41]).

Unlike T cells, B cells can directly recognize antigens *via* localized immunoglobulins (Igs) on the cell surface called B cell receptors (BCRs) (signal I; B cell signaling

reviewed in Yuseff *et al.*[42]). While B cells can encounter antigens in the periphery, coincidental interactions are likely rare events. Instead, antigens trafficked to B cell zones (follicles) are retained for extended time periods by a network of follicular DCs (FDCs).[43] This temporal and spacial co-localization significantly increases the likelihood of naïve B cells to engage with their cognate antigen. At least two major pathways of antigen delivery to FDCs have been identified. Antigens are captured by SCS macrophages and imported from the SCS into the B cell folllicle.[44,45] Here, antigen can either be recognised by cognate B cells, or relayed by non-cognate B cells to FDCs *via* a mechanism dependent upon complement and complement receptor 2 (CD21).[46] Alternatively, protein antigens with a hydrodynamic radius around 4-5 nm (M_w ~70 kDa) may diffuse directly *via* conduits from the SCS to the B cell follicle.[47] BCR binding to cognate antigens triggers internalization, B cell activation, upregulation of antigen processing machinery, and presentation of degraded antigens *via* MHC class II.[48,49] Activated B cells migrate to the T cell zone/B cell zone border where TCR:MHC II interactions with antigen-specific CD4+ T cells leads to the provision of T cell "help" *via* CD40:CD40 ligand (CD40L) signalling (signal II).[50,51] This in turn promotes the upregulation of transcription factor Bcl-6 in both B cells and T cells,[52-54] driving the formation of germinal centers in B cells, which are specialized foci of B cell proliferation and maturation (reviewed in Victora *et al.*[55]). Germinal centers function as the site of BCR diversification and enable the process of affinity maturation, whereby B cells are selected for high affinity binding to cognate antigens by sequential rounds of proliferation and competition for limited CD40L-dependent help from T follicular helper (Tfh) cells.[56] B cells exiting germinal centers can differentiate into long lived memory B cells that circulate in the periphery. A subset of

generally high affinity B cells selected in the germinal center initate a differentiation program toward plasma cells, which are highly specialized for the secretion of antibodies, the soluble secreted forms of the BCR. Plasma cells migrate *via* the bloodstream and take up long-term residence within bone marrow niches where they can provide a stable and long-term source of serum antibodies, for some antigens up to the lifetime of the host. Antibodies can mediate direct neutralization of pathogens and/or the clearance of infected cells *via* mechanisms such as antibody-dependent cellular cytotoxicity (ADCC)[57] or antibody-dependent phagocytosis (ADP).[58,59]

Recapitulating the complex coordination of immune cells required for the generation of an efficacious adaptive immune response is a challenge for vaccine development. However, an ever expanding understanding into the immunological principles driving vaccine immunogenicity creates opportunities to harness complex immune systems with rationally designed, next-generation vaccines and thereby maximize protective potential.

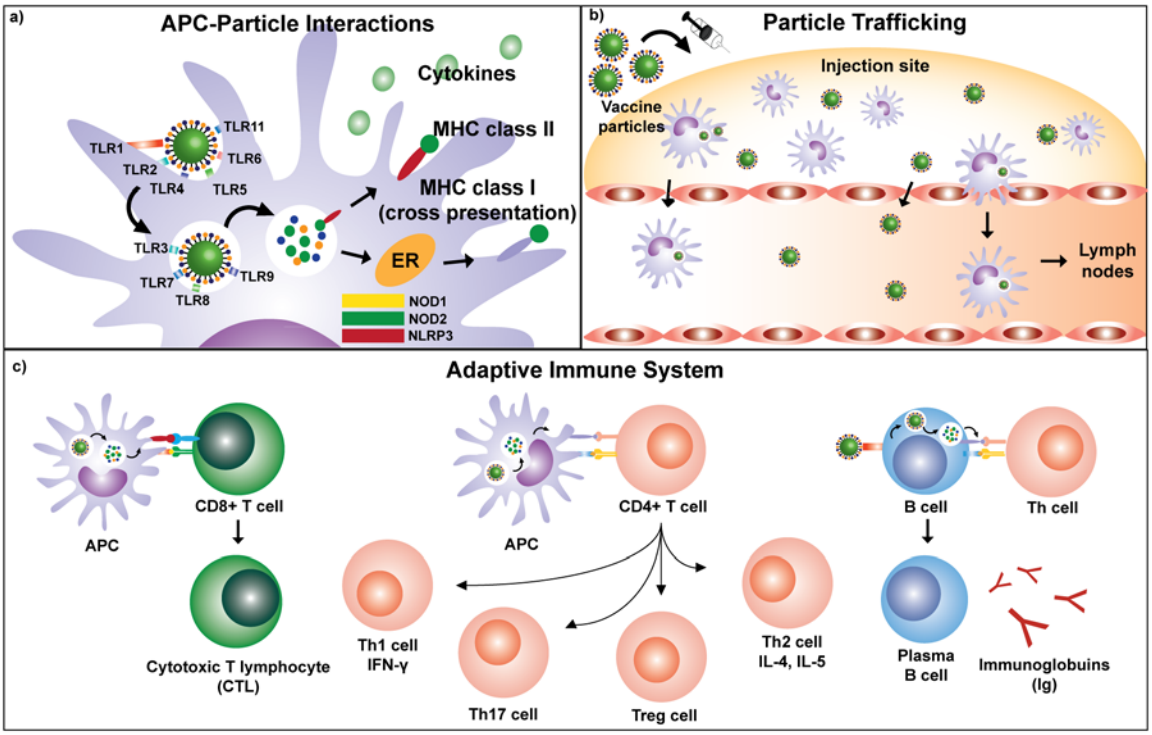

Figure 1. Vaccine administration and induction of innate and adaptive immune responses. a) Vaccines are administered via intramuscular (most common), intradermal, oral, and mucosal routes, where they encounter local immune cells such as neutrophils, monocytes, macrophages, and dendritic cells (DCs), a subset of antigen presenting cells (APCs) highly specialized for antigen capture and presentation. Upon internalization, vaccine particles can activate PRRs on the cell surface (e.g., TLR1, TLR2, TLR4, TLR5, TLR6, TLR11), endosome (e.g., TLR3, TLR7, TLR8, TLR9), and cytoplasm (e.g., NOD1, NOD2, NLR3). Captured vaccines are degraded with endosomal/lysosomal compartments into peptide fragments, which are sucsequently presented on the cell surface upon major histocompatibility complex (MHC) molecules. b) Internalization of antigen and the engagement of pattern recognition receptors (PRRs) induces DC maturation, which facilitates migration out of the muscle to lymphoid organs via the blood or lymphatic system. Small vaccine particles (~20-30 nm) can effectively traffic to

lymph nodes via convective flow without assistance from migratory APCs at the site of administration; whereas larger particles are more likely to be retained at the injection site and require transport into the lymph nodes by migratory APCs. c) DCs activate CD8+ and CD4+ T cells via MHC class I and II presentation, respectively. Activated CD8+ T cells can differentiate into cytotoxic T lymphocytes (CTLs), which are crucial for control against intracellular pathogens and cancer. Activated CD4+ T cells can differentiate into T helper (Th) cells, such as Th1 (IFN-γ-producing), Th2 (IL-4- and IL-5-producing), Th17, or regulatory T cells (Treg) that provide critical support to other immune cells, such as CTLs, via complementary cytokine secretion, and to serum antibody responses, via CD40:CD40 ligand co-stimulation of antigen-specific B cells.

MODULATION OF VACCINE IMMUNE RESPONSES BY CELLULAR RECEPTORS FOR MICROBIAL COMPONENTS

A critical role of the innate immune system is to scan foreign material and relay critical information to the adaptive immune system to modulate the strength and quality of protective immunity.[19,60,61] In general, this occurs through activation of PRRs. A range of PRR agonists are now under intense investigation for use as adjuvants that target specific innate immune cell recognition pathways.[62,63] For example, Monophosphoryl lipid A (MPL) was the first PRR agonist approved for use in human vaccines and many others are undergoing preclinical and clinical trials.[64,65] MPL is a derivative of lipid A from *Salmonella minnesota* R595 that is detoxified by mild hydrolytic treatment. MPL has been formulated with alum in an adjuvant called AS04 that is licensed for use in HPV

and HBV vaccines. AS04 is considered safe and more effective than alum,[66] thus solidifying the potential of PRR agonist-based adjuvants. Studies clearly indicate that activation of PRRs has varied and complex effects on the outcome of immunization,[19] which can be exploited in rational adjuvant design. It should be noted that improved adjuvants using PRR and other approaches often results in increased local and systemic side effects (increased reactogenicity). Although some side effects will be tolerable in the setting of a high risk of acquisition of severe diseases, an important goal of PRR-adjuvant vaccine research is to improve immunogenicity without unacceptable increases in side effects. TLR and NOD-based approaches are among promising adjuvants in this regard.

Toll-like Receptors. Toll-like receptors (TLRs) are the most extensively characterized PRRs with 10 and 13 TLRs identified in humans and mice, respectively. TLRs on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) recognize bacterial products such as lipopolysaccharide (LPS), lipoteichoic acids, lipoproteins, and flagellum. Endosomal TLRs (TLR3, TLR7, TLR8, TLR9) recognize viral or bacterial nucleic acids, which can be accessed during viral replication or upon intracellular degradation. TLR activation mainly polarizes Th1-biased adaptive immune responses;[19,35,67-69] however, TLR2 activation has been shown to polarize Th2-biased immune responses.[70,71] There is also a clear trend in several studies showing that endosomal TLR signaling enhances cross presentation and CD8+ T cell responses,[72-79] and that surface TLRs can actually suppress CD8+ T cell responses.[79]

Activation of multiple TLRs can result in synergy or inhibition of immune responses *via* intracellular crosstalk, the mechanisms of which have been reviewed in detail.[80-82] Various reports have shown that TLR pathways that use the adapter molecule, MyD88

(all TLRs except TLR3), can synergize with TLR pathways that signal through the adapter molecule, TRIF (TLR3 and TLR4) in the induction of innate inflammatory responses,[83-86] Th1 polarization capacity,[84] and antibody responses.[87] Zhu *et al*. demonstrated that the combination of TLR3, TLR9, and TLR2/6 ligands induced CD8+ T cell responses with synergistically enhanced functional avidity compared with single and paired ligands; however, the number of activated CD8+ T cells was not significantly different.[88] Additionally, immunization with the triple ligand combination signficantly enhanced protection against viral challenge compared with single and paired ligands. Overall, the study demonstrated that even though MyD88-dependent pathways are not synergistic as a pair, when costimulated with TRIF-dependent TLR3, protection can be enhanced through the quality, and not quantity, of the CD8+ T cell responses.

NOD-like Receptors. Up to 22 NOD-like receptors (NLRs) have been identified in humans. Although the triggers and functions of many NLRs remain unknown, NOD1, NOD2, and NLRP3 are the best characterized.[89-91] NLRP3 is a widely studied NLR that senses cellular damage and stress.[92] NLRP3 (and some other NLRs) activate multiprotein complexes called inflammasomes that facilitate the production and release of inflammatory cytokines, IL-1β and IL-18.[93] Activation of the transcription factor nuclear factor-κB (NF-κB) induces transcription for pro-IL-1β while pro-IL-18 is constitutively expressed and increases in expression upon cellular activation. Activated inflammasomes then recruit caspase-1, which is a cysteine-aspartic acid protease that cleaves and activates pro-IL-1β and pro-IL-18 into their bioactive forms.[94,95] It has been shown that alum adjuvants and other particulates (*e.g.*, nanoparticles and microparticles) activate the

NLRP3 inflammasome through lysosomal destabilization, which causes leakage of proteolytic enzymes into the cytosol.[96-98]

NOD receptors (*i.e.*, NOD1, NOD2) recognize peptidoglycan (PGN). NOD2 detects muramyl dipeptide (MDP), which is a motif common to both Gram-positive and Gram-negative bacterial PGN.[99] Notably, MDP is also recognized by NLRP3.[100] NOD1 specifically detects γ-glutamyl diaminopimelic acid (iE-DAP), a breakdown product of PGN, which is found almost exclusively in Gram-negative bacteria.[101,102] Immunization with NOD1 and NOD2 agonists (FK156 and MDP, respectively) with the model protein antigen ovalbumin (OVA) was shown to induce strongly polarized Th2 adaptive immune responses and no CD8+ T cell responses.[103,104] CFA is heat killed mycobacteria that contains agonists for both TLRs and NODs. The same studies showed that optimal Th1, Th2, and CD8+ T cell responses to CFA relied on NOD1 and NOD2 signaling, indicating that NOD signaling can facilitate TLR-driven Th1 and CD8+ T cell responses.[103,104] In contrast, NOD signaling due to PGN contaminants in LPS (TLR4 agonist) was recently found to inhibit cross presentation.[105] Another recent study found that immunization with NOD1 and NOD2 agonists resulted in enhanced cross-presentation *in vitro* and CD8+ T cell responses *in vivo*.[106] Thus, the role of NOD signaling in activating CD8+ T cell responses remains largely unclear, both in the presence and absence of TLR costimulation.

Recently, Pavot *et al*. reported an investigation of a NOD/TLR adjuvant system using a chimeric ligand containing a NOD2 and TLR2 agonist.[107] The chimeric ligand synergistically enhanced Th1-polarized IgG1 and IgG2a production following subcutaneous administration; while single ligands did not signficantly enhance the

antibody response. Several studies have also demonstrated enhanced and synergistic activation induced by signaling between TLRs and NOD receptors.[85,107-111] In our recent study, using a particle-based system, we showed that NOD2 activation played different roles in modulating the adaptive immune response depending on coactivation of TLR9.[112] Specially, NOD2 activation alone resulted in Th2-polarized CD4+ T cell and serum antibody responses; however, in the presence of TLR9 costimulation, there was an enhancement of Th1-polarized CD4+ T cell and serum antibody responses compared with TLR9 stimulation alone. Notably, NOD2 coactivation also abrogated the CD8+ T cell response observed in groups where TLR9 alone was activated.

PARTICLE-BASED VACCINE DELIVERY SYSTEMS

Particulate systems are inherently more immunogenic than soluble systems (e.g., cross-presentation efficiency[113-115]), as nano- and microparticles mimic the size, geometry, and properties that the immune system recognizes. Thus, delivery of subunit antigens using particle-based delivery systems can lead to significant improvements in immmunogencity.[6,8] Virus-like particles (VLPs) were the first subunit antigen- and nanoparticle-based vaccines to reach the market with the FDA approval of the recombinant hepatitis B surface antigen (HBsAg) vaccine in 1986.[116,117] VLPs are self-assembling nanoparticles composed of viral capsid proteins that mimic viral structure but do not contain genetic material. There are now four VLP vaccines on the market: GlaxoSmithKline (GSK)'s Engerix® for hepatitis B virus (HBV) and Cervavix® for human papillomavirus (HPV), and Merck and Co., Inc.'s Recombivax HB® for HBV and

Gardasil® for HPV. There are also many other VLP vaccines currently undergoing preclinical and clinical development.[118]

In addition to VLPs, many other types of particles are under investigation for subunit antigen delivery, including those based on lipids, synthetic polymers, natural polymers, and inorganic materials.[8,119-121] Liposomes are the most widely implemented particle-based system in the clinic and on the market so far. Liposomes are comprised of concentric phospholipid bilayers that contain hydrophilic domains in the interior and exterior and hydrophobic domains in the lipid bilayer.[122] Two liposomal vaccine systems are currently approved for use in humans: Crucell's Inflexal V® for seasonal influenza[123] and Epaxal® for hepatitis A.[124]

Aside from the inherent immunogenicity associated with particulate structure, the properties of particulate delivery systems can be engineered to enhance immune responses through controlled composition (*e.g.*, targeting and/or immunostimulating ligands, multiple antigens[125]) and physicochemical properties (*e.g.*, size, shape, surface properties, degradability).[20,126,127] It is clear that particle properties influence immune responses;[20,126-131] however, a more complete understanding of how to engineer intrinsic particle properties to optimize and/or tune the vaccination outcome is required. The following sections describe studies elucidating the impacts of particle properties on various types of immune responses that are relevant to the outcome of vaccination (*i.e.*, innate immune cell activation, MHC class I antigen presentation, MHC class II antigen presentation, lymph node trafficking, CD4+ T cell responses, CD8+ T cell responses, and B cell responses).

Influence of Particle Size. Particle size plays a significant role in vaccine efficacy due to its influence on lymph node trafficking and localization,[27,132-135] adaptive immune responses,[136,137] and cross-presentation.[138,139] Studies have suggested that vaccine particles approximately 20-30 nm in size can effectively traffic to lymph nodes within 2 h *via* convective flow from the interstitium without assistance from migratory APCs at the site of administration; whereas larger particles are more likely to be retained at the injection site and require transport into the lymph nodes by migratory APCs.[27] For example, Reddy *et al*. showed that 20-25 nm particles entered the dermal lymphatic capillary network and localized in lymph nodes more efficiently than 45 or 100 nm particles.[132,133] Size has also been shown to influence the cellular distribution of particles within the lymph node. For example, Manolova *et al*. showed that upon injection into mice, 20 nm polystyrene beads localized in the SCS and B cell areas while larger particles were excluded from the SCS and found in areas more distal from B cell follicles.[27] In contrast, other studies employing state-of-the-art visualization techniques have suggested that small (40 nm), intermediate (200 nm), and large (1 μm) particles can all directly access lymph nodes *via* the afferent lymphatics,[33] as can bacteria and viruses during infection.[140,141] Therefore, the influence of injection site, local hydrodynamic forces, and particle size on the initiation of immune responses to particle-based vaccines require further investigation.

In terms of immunogenicity, Fifis *et al*. found using different sizes of polystyrene beads with conjugated OVA (20, 40, 100, 200, 500, 1000, 2000 nm), that 40 nm beads induced the highest IFN-γ-producing CD4+ and CD8+ T cell responses and IgG production following intradermal immunization.[136] Compared with 20 nm and 1000 nm

beads, 40 nm beads were associated with a significantly higher percentage of lymph node cells. Out of the OVA-positive lymph node cells, 40 nm beads associated mostly with DCs, whereas 1000 nm beads associated mostly with macrophages. Additionally, 40 nm beads protected against tumor challenge more effectively than 1 μm beads and soluble OVA. A follow-up study compared OVA-conjugated polystyrene beads in a narrower size range (20, 40, 49, 67, 93, 101, 123 nm), showing optimal IFN-γ-producing CD4+ and CD8+ T cell responses upon intradermal immunization with 40 and 49 nm particles (Figure 2a,b).[142] Interestingly, the study also demonstrated significantly higher IL-4-producing CD4+ T cell activation in response to larger beads (93, 101, 123 nm) (Figure 2c). Notably, the study showed minimal differences in IgG production and dominance in the IgG1 isotype across the range of particle sizes. The findings demonstrate the possibility of tuning particle size to polarize CD4+ T cell responses. Another study recently compared the antibody responses induced by gold nanoparticles conjugated with antigenic peptides of 2, 5, 8, 12, 17, 37, or 50 nm, showing that 8 nm nanoparticles induced the highest levels of antibody production, while the 37 and 50 nm nanoparticles were ineffective.[137]

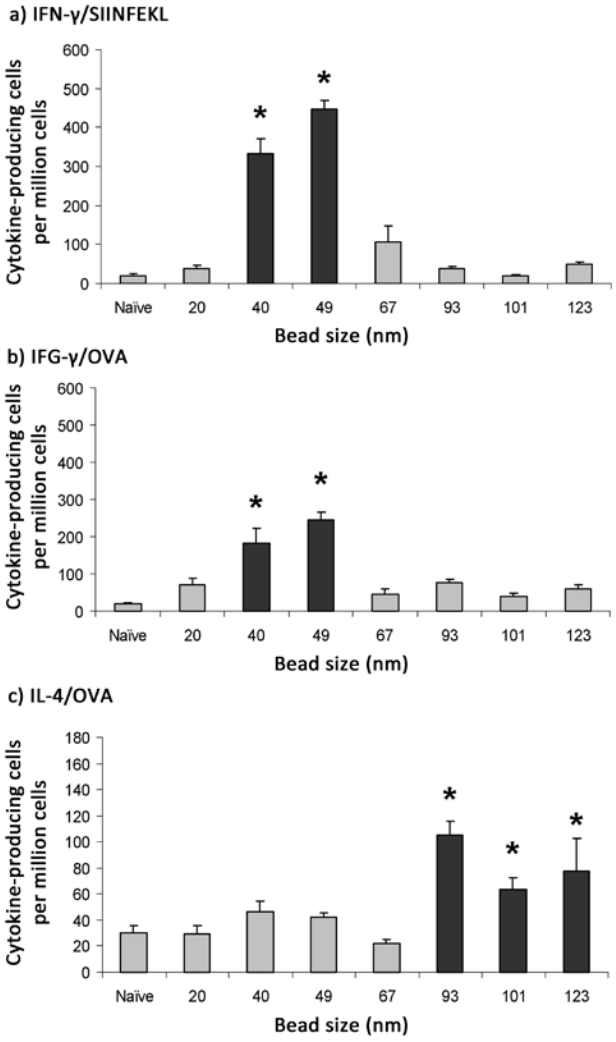

Figure 2. Impact of particle size on T cell immunogenicity in vivo. OVA-conjugated polystyrene particles 40 and 49 nm in diameter induce CD8+ T cell (a) and IFN-γ-producing CD4+ T cell responses (b); whereas 93, 101, and 123 nm particles induce IL-4-producing CD4+ T cell responses (c). Adapted from ref 142. Copyright 2007 American Chemical Society.

Regarding the effect of particle size on cross-presentation efficiency, studies indicate that decreased particle size is correlated with increased efficiency of cross-presentation.[138,139] For example, Hirai *et al*. compared the cross-presentation efficiency of DCs pulsed with different sized silica particles (70, 100, 300, 1000 nm) and OVA.[138] The study showed that 70 and 100 nm particles enhanced antigen localization in the cytosol from endosomes and induced cross-presentation, while 300 and 1000 nm particles did not.

Influence of Particle Shape. Studies have indicated that shape may critically influence the efficacy of particle-based systems used for drug and vaccine delivery.[143,144] It is known that shape plays an important role in cellular uptake, as demonstrated in studies showing enhanced internalization of spherical particles compared with particles with high aspect ratios.[145-147] Sharma *et al*. reported that internalization was dependent on cell membrane binding, where longer particles were more efficiently attached, but internalization was inhibited by size.[145] Another study recently showed that rods exhibited higher specific uptake and lower nonspecific uptake compared with spheres conjugated with targeting antibodies.[148] Niikura *et al*. showed that gold rods (40 x 10 nm) were taken up more efficiently than spheres (20 and 40 nm) and cubes (40 x 40 x 40 nm) in both mouse macrophages and DCs.[149] TEM images showed that 20 nm spheres and rods escaped endosomes and localized in the cytoplasm following uptake while 40 nm spheres and cubes remained in endocytic compartments. Additionally, only rods induced significant levels of IL-1β and IL-18 secretion in DCs, indicating activation of the inflammasome, probably through lysosomal rupture during endosomal escape. On the other hand, 40 nm spheres and cubes induced significant TNF-α, IL-6, IL-12, and GM-

CSF secretion. *In vivo*, 40 nm spheres coated with the West Nile Virus envelope protein induced the highest total IgG production in mice compared with rods, cubes and 20 nm spheres. The study showed an inverse relationship between the specific surface area (total surface area per particle volume) and antibody production and TNF-α secretion (Figure 3). As the specific surface area depends on both size and shape, the study indicates that both of these parameters are crucial in determining the immune response.

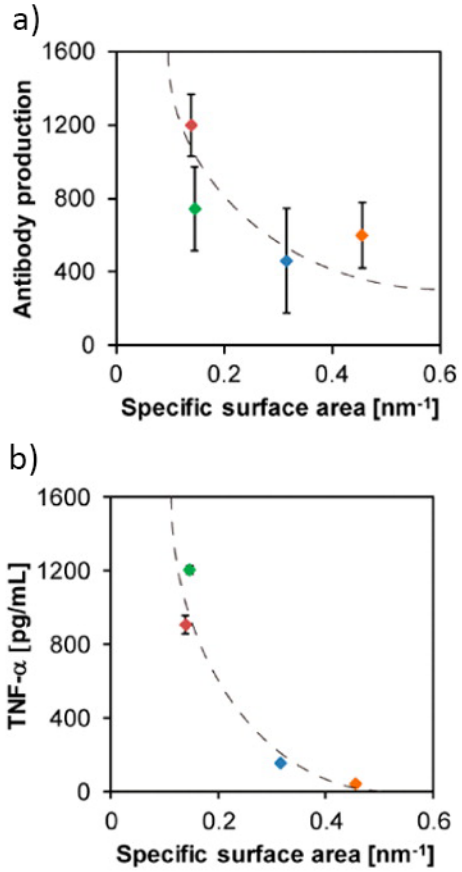

Figure 3. Impact of surface area on immunogenicity. (a) Antibody production or (b) TNF-α secretion by DCs shown as a function of the specific surface area (total surface

area per particle volume) of a given particle vaccine. 20 nm spheres (blue), 40 nm spheres (red), cubes (green), rods (orange). Adapted from ref 149. Copyright 2013 American Chemical Society.

Influence of Particle Charge. It is well known that positive surface charge enhances internalization by cells *via* electrostatic attractive forces between particles and negatively charged cell membranes.[150,151] Positively charged particles are also exploited for enhancing immune responses at mucosal tissues,[152-154] which is required to induce mucosal immunity necessary for pathogens that enter at mucosal surfaces. Following pulmonary immunization, Thomas *et al*. found that positively charged polyethyleneimine (PEI)-modified PLGA microspheres induced higher antibody and T cell responses compared with unmodified particles.[152] Fromen *et al*. compared OVA-conjugated hydrogel nanoparticles that varied in charge but had constant size, shape, and antigen loading.[153] Pulmonary immunization with cationic nanoparticles enhanced systemic and lung antibody titers, germinal center B-cell expansion, and increased CD4+ T cell activation in lung draining lymph nodes compared with anionic nanoparticles. Additionally, DCs treated *ex vivo* with cationic nanoparticles induced enhanced T cell proliferation, expression of MHCII, T cell costimulatory molecules, and cytokine secretion compared with anionic nanoparticles or soluble OVA. Recently, Stary *et al*. showed that by delivering UV-inactivated *Chlamydia trachomatis* (UV-Ct) and R848 (resiquimod), a TLR7/8 agonist, *via* charge switching nanoparticles antigen presentation was redirected to immunogenic DCs, whereas UV-Ct on its own is presented by tolerogenic DCs, causing an exacerbation of host susceptibility in conventional and

humanized mice.[154] These particles had a cationic charge below pH 6.5 (allowing conjugation with negatively charged UV-Ct) and a slight negative charge at physiological pH 7.4.

Influence of Particle Hydrophobicity. Seong and Matzinger proposed that hydrophobicity was one of the signals recognized by the innate immune system.[155,156] In agreement with this notion, various studies have correlated hydrophobic particle properties with enhanced immune responses.[157,158] For example, Moyano *et al*. recently showed that increasing hydrophobicity of surface attached ligands on gold nanoparticles was correlated with upregulation of proflammatory cytokine gene expression.[157] In another study, the effect of microparticle hydrophobicity was evaluated *in vitro* and *in vivo* using particles that were constant in size and morphology but were made from polymers that differed in hydrophobicity: poly(D, L-lactic acid) (PLA), poly(D, L-lactic-co-glycolic acid) (PLGA), and poly(monomethoxypolyethylene glycol-co-D, L-lactide) (mPEG-PLA).[158] The study correlated the increased hydrophobicity of PLA microparticles with increased cellular internalization and upregulation of MHCII and CD86 expression in DCs *in vitro* and significantly elevated IFN-γ- and IL-4-producing T cell responses following subcutaneous immunization. Thomas *et al*. demonstrated that carboxylated nanoparticles induced activated complement *in situ* and enhanced antibody production and T cell responses *in vivo* compared with hydroxylated surfaces.[159] Shahbazi *et al*. showed enhanced immunostimulatory effects *in vitro* and *in vivo* using nanoparticles with high levels of C-H structures on the surface compared to those with nitrogen and oxygen.[160]

A series of studies by the Narasimhan group studied the complex immunological effects of polyanhydride nanoparticles with varied chemistry and hydrophobicity using copolymers based on sebacic acid (SA), 1,6-bis-(p-carboxyphenoxy)hexane (CPH), and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaocatane (CPTEG). The least hydrophobic particles (*i.e.*, SA-rich) were shown to be more efficiently internalized by DCs than the more hydrophobic particles (*i.e.*, CPH-rich).[161] Additionally, the more hydrophobic particles did not induce the production of IL-6, IL-1β, of TNF-α by DCs, but did induce expression of MHC II and CD86. On the other hand, the less hydrophobic particles induced production of higher amounts of secreted cytokines but no expression of surface markers. The molecular descriptors responsible for DC activation patterns were determined using informatics analysis, finding number of backbone oxygen moieties, percentage of hydroxyl end groups, polymer hydrophobicity, and number of akyl ethers to be the most important.[162] The relationship between particle chemistry and the kinetics and maturation of the induced humoral response upon pulmonary immunization of particles containing F1-V antigen was also examined.[163] The least hydrophobic particles (20:80 CPH:SA) degraded the fastest and more rapidly induced an antibody response. CPH-rich formulations (20:80 CPTEG:CPH, 50:50 CPTEG:CPH) degraded more slowly, persisted in the lungs for at least 63 days, and induced higher antibody titers with a greater breadth of epitope specificity. It was hypothesized that the induction of longer lived plasma cells was due to the slow and continuous release of antigen as well as a more inflammatory environment assumed to be induced by the hydrophobic character of the particles.

ADVANTAGES OF PARTICLE-BASED VACCINES OVER TRADITIONAL FORMULATIONS

High Density Array of Vaccine Antigens. In contrast to T cell responses, which require APC intermediaries to initiate a primary immune response, B cells have the capacity to directly engage vaccine antigens. Subunit antigens do not effectively induce an antibody response when injected in their free, soluble state because B cells have evolved to recognize dense, highly repetitive epitope arrangements on the surfaces of pathogens (*e.g.*, viruses, flagellum) or alternatively, arrayed epitopes bound in immune complexes on the surface of FDCs. Highly repetitive arrays of epitopes in vaccines can efficiently crosslink BCRs and trigger potent B cell activation, resulting in enhanced B cell responses. The density and conformation of the encountered antigen can significantly modulate subsequent immunity. A major advantage of particle-based vaccines is the ability to finely control these aspects of antigen delivery. For example, Kanekiyo *et al*. showed that an epitope presented by self-assembling nanoparticles of ferritin (octahedral cage consisting of 24 subunits) or encapsulin (icosahedron made of 60 identical subunits) resulted in significantly enhanced antibody titres compared with the soluble epitope.[164] Using VLPs with covalently attached epitopes of different density, Jegerlehner *et al*. showed that the magnitude of antibody responses was significantly correlated with epitope density.[165] The study showed that 60 epitopes per particle spaced 5-10 nm apart drove maximal humoral immune responses following immunization of mice. Paus *et al*. showed that antigen density on sheep red blood cell conjugates was crucial for activating the extrafollicular plasma B cell response but not the germinal center response.[166] Some small moieties (termed “haptens”) are not immunogenic unless conjugated to a larger

carrier (usually protein). This is especially relevant for bacterial polysaccharides, which require protein conjugates for vaccine efficacy, such as those used in medically important *Haemophilus influenzae* type B, meningococcal, or pneumococcal vaccines. While nanoparticles can directly substitute for the protein carrier in some cases to increase the immunogenicity of haptens,[167] protein-based nanoparticles may offer the ability to act as effective protein carriers for hapten-based vaccines.

Codelivery of Adjuvants and Immunomodulatory Agents. Immunostimulating ligands can be simultaneously delivered with vaccine antigens to enhance vaccine efficacy, with co-packaging of both a means to maximize delivery to the same immune cells *in vivo* and thereby limit off target adjuvant affects. This is particularly important for the safety of PRR agonists, as it spatially constrains the action of PRR agonists and avoids nonspecific inflammatory responses. A number of studies have shown that the attachment of immunomodulatory agents, such as PRR ligands,[87,112] DC-targeting antibodies,[168] ER-targeting peptides (for enhancing cross-presentation),[169] and PEG,[170] can enhance and tune immune responses. Ligands can be incorporated into particles by encapsulation, physical adsorption, or covalent conjugation.[171-173] Covalent conjugation is the preferred method for incorporating PRRs agonist and other biofunctional ligands due to controllability over ligand density and orientation. A variety of coupling techniques have been established for ligand conjugation.[174]

Recently, studies have emerged demonstrating copackaging of multiple PRR agonists within a single particle.[87,112,175] Using a particle-based delivery system, Kasturi *et al*. found that immunization of mice with synthetic nanoparticles containing antigens and TLR4 (MPL) and TLR7 (R837) ligands induced synergistic increases in antibody

production that depended on direct TLR4 and TLR7 activation on the same B cell (Figure 4).[87] Notably, however, human B cells do not constitutively express TLR4, and so the implications of TLR4/7 co-signaling are not clear for human vaccines. In our recent study, a mesoporous silica-templated protein antigen (OVA) particle was covalently conjugated with either NOD2, TLR9, or a combination of both ligands leading to qualitatively and quantitiavely different innate and adaptive immune responses.[113]

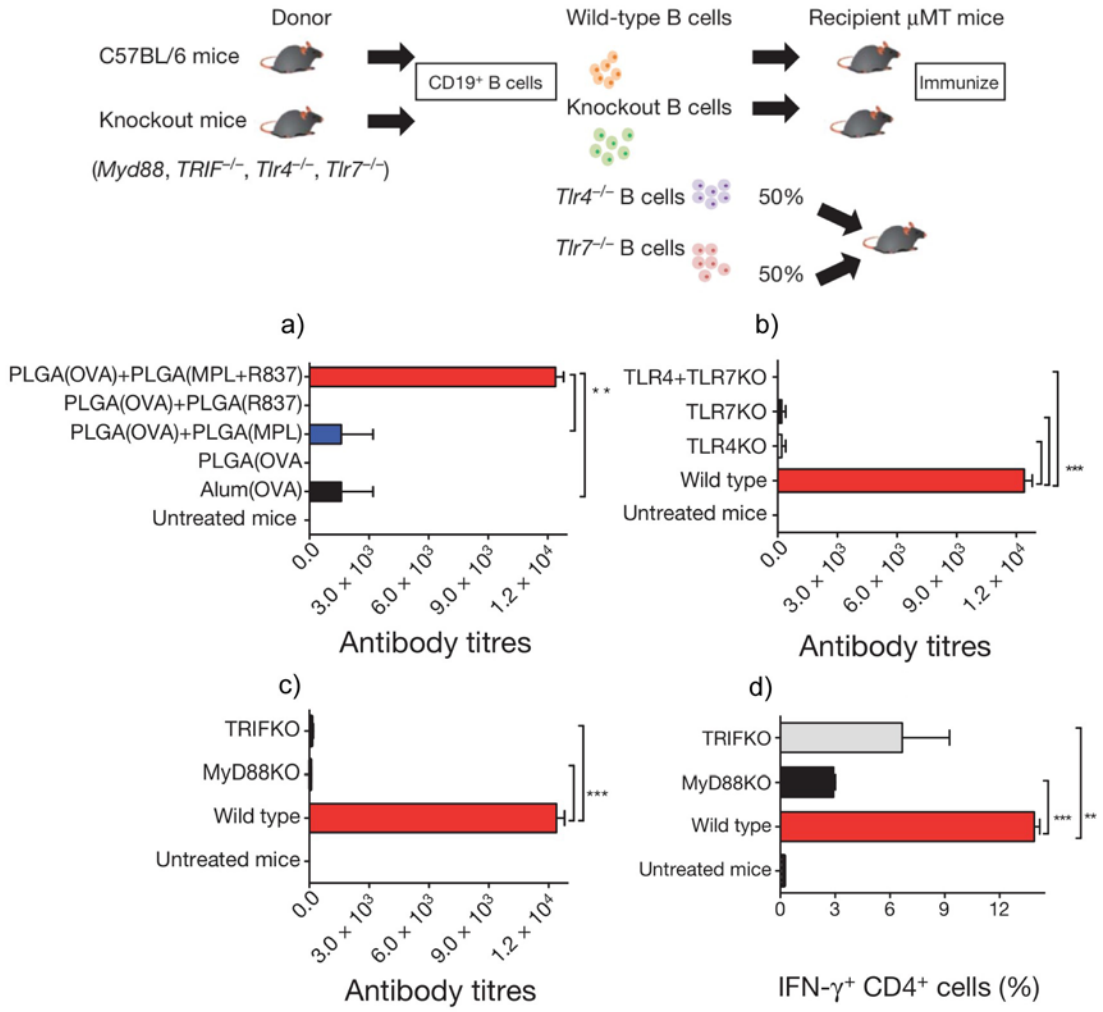

Figure 4. Codelivery of MPL and R837 drives TLR4 and TLR7 activation, respectively, on the same B cell, leading to synergistic antibody production. a) B cell-deficient mice

(μMT mice) reconstituted with B cells from TRIF-/-, MyD88-/-, TLR4-/-, and/or TLR7-/- mice. b) Synergy is replenished in μMT mice reconstituted with B cells from wild-type mice. c) Antibody responses are diminished in μMT mice reconstituted with B cells from TLR4-/- mice, TLR7-/- mice, or a 1:1 mixture of both. d) Antibody responses are diminished in μMT mice reconstituted with B cells from TRIF-/- or MyD88-/- mice. e) CD4+ T cell responses are substantially reduced in μMT mice reconstituted with B cells from TRIF-/- or MyD88-/- mice. Adapted from ref 87. Copyright 2011 Macmillan Publishers Ltd.

The density of surface ligands, has also been correlated with particle immunogenicity.[176] OVA-containing PLGA nanoparticles functionalized with avidin-palmitic acid were surface modified with varying amounts of biotinylated anti-DEC-205 monoclonal antibodies (Figure 5).[176] The amount of IL-10 produced by DCs *in vitro* and IL-10 and IL-5 produced by CD4+ T cells upon restimulation *in vitro* increased with ligand density. These results were shown to be independent of DC uptake. Particles were also used to boost the primary immune response to OVA in CFA to determine whether this trend was reproduced *in vivo*. The results showed that IL-10 and IL-5 secretion by splenocytes restimulated with OVA also increased with increasing ligand density. This effect was shown to be due to variations in receptor crosslinking.

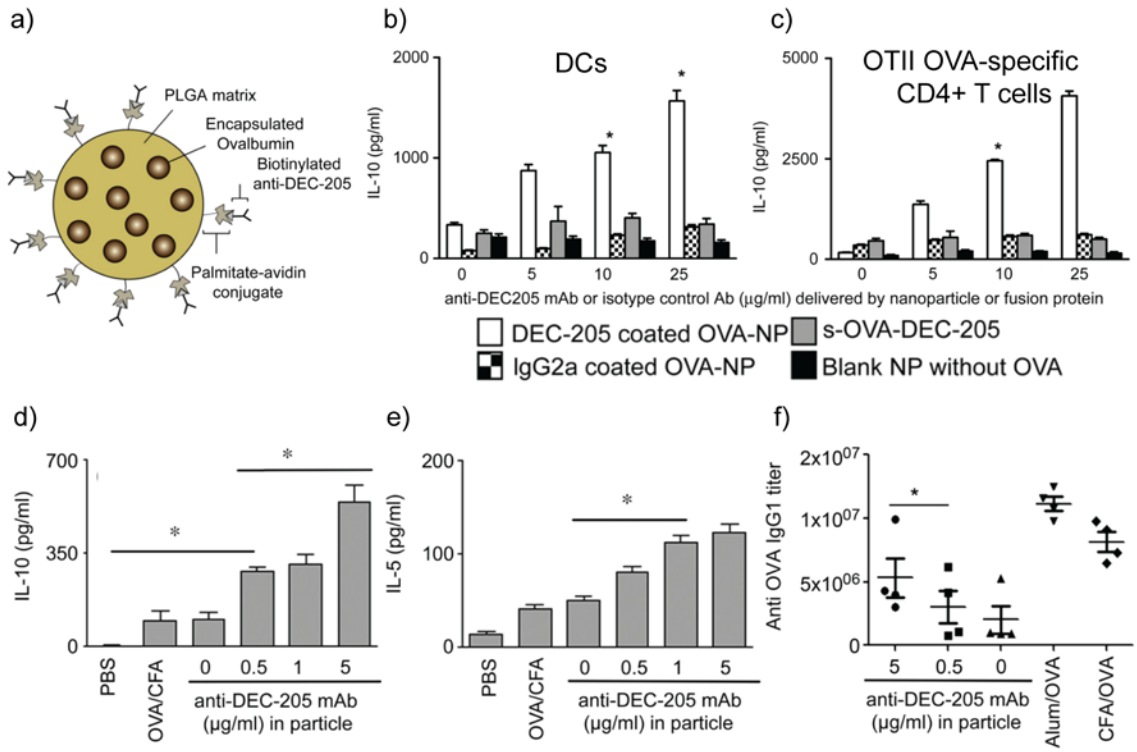

Figure 5. Antibodies targeting antigen to immune cells PRRs influences immune responses in vitro and in vivo. a) OVA-encapsulated PLGA particles with anti-DEC205 monoclonal antibody conjugated via avidin-biotin; b) IL-10 secretion from DCs incubated with indicated particles or soluble OVA with DEC205 conjugate; c) IL-10 secretion from OVA-specific CD4+ OTII T cells incubated with DCs from (b) for 72 h; d-e) IL-10 and IL-5 secretion from whole splenocytes restimulated with OVA following booster immunization with indicated groups; f) IgG1 titre following intraperitoneal immunization with indicated groups. Adapted from ref 176. Copyright 2011 Elsevier.

Controlled Rates of Intracellular Cargo Release. For the generation of CD8+ T cell responses, particle-based antigens must be cross-presented by APCs *via* MHC class I.

Thus, the controlled release of encapsulated antigens upon intracellular degradation is a widely implemented approach to enhance cross-presentation. Various strategies have been proposed for engineering intracellular stimuli-responsive release mechanisms in particles such as systems based on pH,[177-179] redox,[180-182] and enzymatic activity.[183-185] A study by Howland *et al*. demonstrated the dependence of antigen release kinetics on MHC class I presentation efficiency, using yeast cells with surface-displayed model antigen peptides constructed by fusing peptides to receptors on the yeast cell membrane *via* disulfide bonds.[186] Release kinetics were manipulated by including linkers of varying proteolytic degradability. When the yeasts were incubated with DCs, the pattern of cross-presentation was similar to the pattern of protease cleavage, indicating that faster antigen release within the phagosome results in more efficient cross-presentation. The study also showed that antigen released beyond 25 min did not significantly contribute to cross-presentation, suggesting a limited window for productive intracellular antigen release, and that antigen released after 25 min may be mostly degraded by lysosomal proteases. In another study, Broaders *et al*. compared antigen presentation induced by dextran microparticles with tunable degradation rates based on modification of the dextran with acetal groups (Figure 6).[187] Acid-catalyzed hydrolysis of the acetals regenerates native dextran and acetone and methanol by-products. The study showed that particles that degraded more rapidly (*i.e.*, low acetalation) induced significantly better MHC class I and MHC class II antigen presentation.

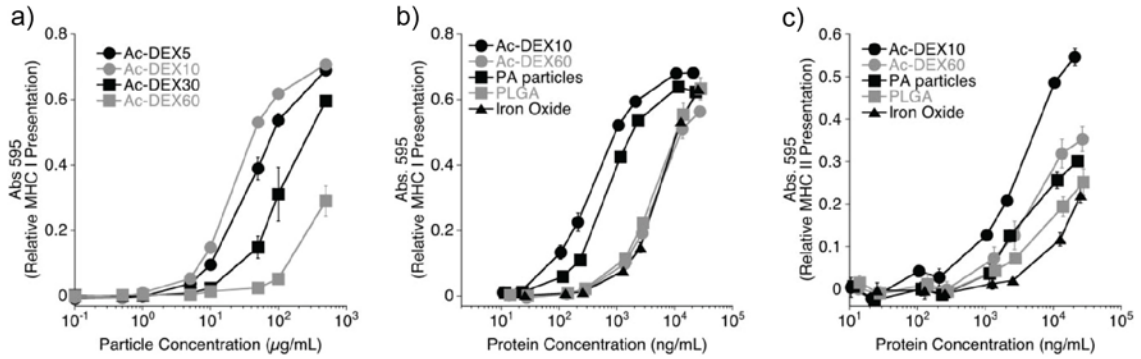

Figure 6. Enhanced MHC class I and class II antigen presentation is correlated with rapid intracellular antigen release kinetics. Adapted from ref 187. Copyright 2009 National Academy of Sciences.

Also using acetalated dextran particles with encapsulated polyIC (TLR3 ligand), Peine *et al*. found that low acetalation (*i.e.*, rapid degradation) was correlated with enhanced cytokine secretion (*i.e.*, IL-1β, IL-2, IL-6, TNF-α, IFN-γ) by a DC-like cell line.[188] In contrast, IL-12 showed an inverse correlation. Although the reasons behind this trend are not clear, the study indicates that the release rate of PRR agonists in particle-based systems influences T cell-polarizing inflammatory responses.

CONCLUSIONS AND OUTLOOK

Particle-based systems have tremendous potenetial for enhancing vaccine immunity, with the option of targeting *in vivo* and the codelivery of multiple antigens and adjuvant ligands. Several recent studies have emerged elucidating key parameters that govern vaccination outcome by particle-based systems. As our understanding of these principles

grows, the rational improvement of synthetic particle-based vaccines will rely on elegant studies that focus on filling crucial knowledge gaps.

Vaccine formulations that enhance Th1 responses, CD8+ T cell responses, and mucosal immunity are currently highly sought after for effective immunization against pathogens for which there are not currently licensed vaccines. Thus, developing improved approaches for polarizating CD4+ T cell differentiation, enhancing cross-presentation, and navigating the mucosal barrier are currently the focus of many efforts. To meet these goals, a clearer understanding of how to rationally formulate particle-based vaccines will be needed. As induced immune responses are a complex interplay of many particle characteristics, as well as other immunization conditions (*e.g.*, route of administration, booster injections, age and health of recipient), accurate predictions of vaccination outcomes will likely require multiparameter models, which have recently emerged for correlating particle properties with blood protein adsorption, cellular internalization, and cell viability.[189,190] It is expected that these types of multiparameter models will provide important insights moving forward. The rational design of particles for highly specific and robust immunity provides an exciting path for the generation of vaccines for which effective immunization schemes are currently lacking.

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Notes

The authors declare no competing financial interest.

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