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Nanomaterial-Based Vaccine Adjuvants

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Abstract

Vaccination is a biological process that administrates antigenic materials to stimulate an individual's immune system to develop immunity to a specific pathogen. It is the most effective tool to prevent illness and death from infectious diseases or diseases leading to cancers. Because many recombinant and synthetic antigens are poorly immunogenic, adjuvant is essentially added to vaccine formula that can potentiate the immune responses, offer better protection against pathogens and reduce the amount of antigens needed for protective immunity. To date, there are nearly 100 different types of adjuvants associated with about 400 vaccines that are either commercially available or under development. Among these adjuvants, many of them are particulates and nano-scale in nature. Nanoparticles represent a wide range of materials with novel physicochemical properties that exhibit immunostimulatory effects. However, the mechanistic understandings on how their physicochemical properties affect immunopotentiation remain elusive. In this article, we aim to review current development status of nanomaterial-based vaccine adjuvants, and further discuss their acting mechanisms, understanding of which will benefit the rational design of effective vaccine adjuvants with improved immunogenicity for prevention of infectious disease as well as therapeutic cancer treatment.

Abstract

Engineered nanomaterials as vaccine adjuvants are capable of potentiating the immune responses through different mechanisms.

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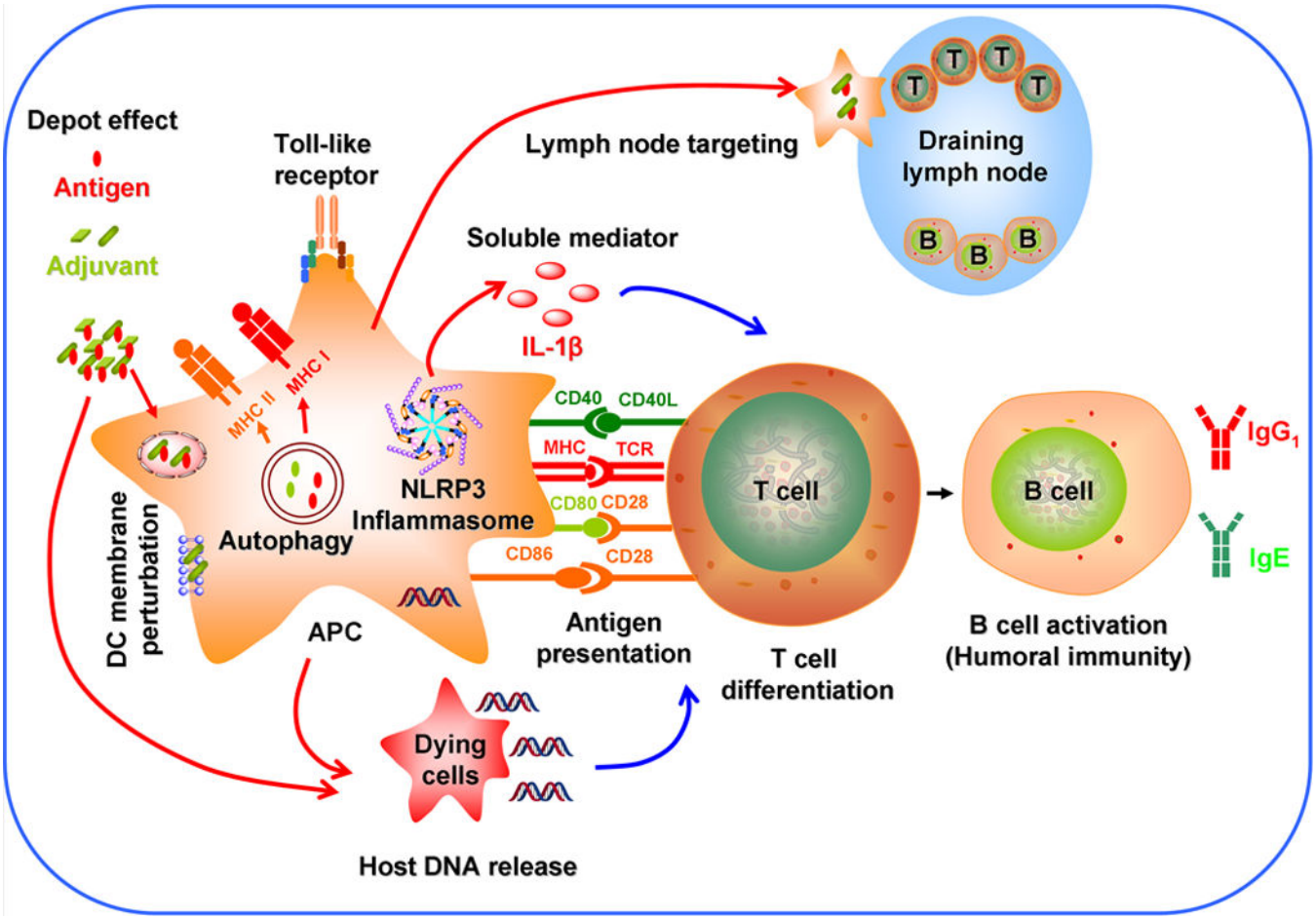

1. Introduction

Vaccination remains one of the most effective tools to stimulate protective immune responses against infectious disease.[1–3] Since Edward Jenner's use of cowpox materials to provide protection against smallpox in 1796, vaccination has saved millions of lives. It has completely eliminated smallpox, the near-complete eradiation of poliomyelitis, and a significant decrease in the incidence of diseases including diphtheria, tetanus, pertussis, measle, hepatitis A, hepatitis B, *etc*.[4] In addition, vaccination can prevent diseases that lead to cancers. The hepatitis B vaccine is 95% effective in infection prevention and the development of chronic disease and liver cancer.[5] Gardasil, a human papillomavirus (HPV) vaccine, can protect against two types of HPV that cause 70% of cervical cancer, 70% of vaginal cancer and 50% of vulvar cancer.[6] Successful vaccines contain not only protective antigens, but also adjuvants that trigger innate and adaptive immune activations for optimal and long lasting immunogenicity.[7] Statistics by Vaxjo, a web- based central database and analysis system that stores vaccine adjuvants and their usages in vaccine development, shows that 93 vaccine adjuvants have been used in 379 vaccines against 78 pathogens, cancers and allergies.[8] Among these, only very few vaccine adjuvants are licensed for use in humans. For example, the Food and Drug Administration (FDA) of the United States

approves four adjuvants including aluminum salts, AS03, AS04, and MF59 (Table 1).[4, 9] In Europe, besides these four adjuvants, virosomes have been licensed and safely used since 1994.[10]

Most of the vaccine adjuvants currently being used or under development are nano-range particulates.[11] Compared to traditional materials, nanomaterials can protect the antigen from the surrounding biological milieu, increase their half-life, minimize the systemic toxicity, promote the delivery of immunomodulatory and immunostimulatory substances to antigen presenting cells, or trigger the activation of antigen-specific T cells.[12] Although particulates are widely used as vaccine adjuvants, the mechanisms of these nano-particulate adjuvants in simulating innate and adaptive immunity are poorly understood.[11] In this review, we aim to introduce current status of the development of nanomaterial-based vaccine adjuvant and to bridge the concept of materials science and immunology. The understanding of immuno-stimulating mechanisms will provide knowledge to design more effective engineered prophylactic and therapeutic vaccine for the prevention and treatment of infectious disease and cancer.

2. Nanoparticles as Adjuvants

Nanomaterials refer to particulate materials having a length scale in the range of 1 nm to 100 nm in at least one dimension.[13] Broader definition raises the range up to 1 μm, based on their similar physicochemical properties to nanoscale particles. They have unique physicochemical properties including size, shape, surface chemistry, roughness and surface coatings that are distinctive from bulk materials, and have been widely used in biomedical applications including drug/gene delivery, vaccines, imaging, and medical devices.[14–21] Among these, nanomaterials is known to generate or enhance immunological responses originate from the interactions at the nano-bio interface.[13, 22, 23] One of the most important advantages of ENMs is that it is possible to control their properties through engineered design, allowing the selection of the most effective adjuvant formulations using *in vitro* and *in vivo* approaches in a systemic fashion. For example, studies have demonstrated that by controlling the physicochemical properties of nanomaterials including the ability to generate ROS, aspect ratio,[24, 25] dispersion state,[26] size[27–29] and surface functionalization,[28–30] it is possible to modulate the immune activation and further enhance immune responses to antigens.[22–31]

3. Engineered Nanomaterials (ENMs) as Vaccine Adjuvants

Nanomaterials are demonstrated to provide adjuvant activity by enhancing the delivery of antigens to the immune system or by potentiating innate and/or adaptive immune responses.[13] Research groups have developed various ENM-based adjuvants to provoke long-lasting immune responses (Table 2). We will discuss the major groups of nanomaterial-based adjuvants and ways to improve the adjuvant effects.

3.1. Metal- and Metal Oxide-Based Vaccine Adjuvants

3.1.1. Aluminum-based Vaccine Adjuvants—Aluminum-based vaccine adjuvants have been in routine use in human vaccination against various diseases including DTaP

(Diphtheria, Tetanus, acellular Pertussis), Human Papillomavirus, Pneumococcal, Hepatitis A and Hepatitis B with safe record for over eighty years (Table 3).[43] Depending on the commercial sources, they are composed of aluminum hydroxide (Alhydrogel), aluminum phosphate (Adju-Phos), aluminum potassium sulfate (Alum), aluminum hydroxyphosphate sulfate (AHSA) or a mixture of aluminum and magnesium hydroxides (Imject Alum)[43–45] These commercially available aluminum salts have different physicochemical properties.[46] For example, aluminum hydroxides are needle-like particles with diameters of 2 nm, while aluminum phosphates are plate-like particles with primary size of 50 nm. However, their names do not correctly describe their adjuvant structures. X-ray diffraction analysis and infrared spectroscopy have identified aluminum hydroxide adjuvant as crystalline aluminum oxyhydroxide, AlOOH; and aluminum phosphate is determined as amorphous aluminum hydroxyphosphate, $Al(0H)_x(P04)_y$.[46] In addition, the key physicochemical properties that determine the nano-bio interaction and the stimulation of immune responses still remain unknown.

Size effects of aluminum salts were studied by Li *et al.* using ovalbumin and *Bacillus anthracis* protective antigen protein as model antigens.[32] Aluminum hydroxide nanoparticles with mean diameters of 112 nm and 9 μm were prepared. It is demonstrated that aluminum hydroxide nanoparticles (~112 nm) exhibited more potent antigen-specific antibody response than that of the micro-sized (~9 μm) particles. The stronger adjuvant activity of nano-sized particles was correlated with their ability to more effectively facilitate the uptake of the antigens.

Effects of surface coating was studied by Wang *et al.* who prepared phospholipid bilayer-coated aluminum nanoparticles (50 nm in size) through chemisorption, and compared their adjuvant effects with naked particles. The coated adjuvant was more readily taken up by antigen-presenting cells and could induce robust antigen-specific humoral (antibody production) and cellular (cytokine production, *e.g.,* IFN-γ in splenocyte supernatants) immunoresponses with less local inflammation.[48]

Recently, in a study by us, we elucidated the role of shape and aspect ratio in aluminum oxyhydroxide-induced adjuvant effects (Figure 1).[31] A comprehensive library of γ-phase aluminum oxyhydroxide nanoparticles (γ-AlOOH, boehmite) with variation in shape, crystal structure, and surface hydroxyl groups was established using a hydrothermal method. It is demonstrated that shape, crystallinity and hydroxyl content of AlOOH nanoparticles played important roles in the induction of immune responses *in vitro* and *in vivo.* AlOOH nanorods induced NLRP3 inflammasome activation and IL-1β production that was dependent on their hydroxyl contents and reactive oxygen species (ROS) generation ability in both THP-1 cells and bone marrow-derived dendritic cells (BMDCs) *in vitro.* AlOOH nanorods also induced the maturation of BMDCs. By using ovalbumin (OVA) as a model antigen, it is shown that vaccination with AlOOH nanorods exhibited higher antibody production including OVA specific IgG_1 and IgE in mice compared to commercial Imject Alum. Adoptive DC transfer was also used to demonstrate that *ex vivo* boosting of APC activity predicts the ability of AlOOH nanorods to exert an adjuvant effect in intact animals. This study shows that the intrinsic properties of AlOOH nanorods play an important role in inducing NLRP3 inflammasome activation that correlates well with the *in vivo* immune-

potentiating effects, which suggests a potential mechanism involved in aluminum-based adjuvants. More research is needed on understanding the relevant mechanisms of other types of aluminum-based adjuvants, which may activate similar pathways or through other mechanisms.

3.1.2. Gold Nanoparticles—Gold nanoparticles (AuNPs) with controlled physicochemical properties are the subject of intensive studies and applications in biology and medicine.[33, 49, 50] Niikura *et al.* investigated the effect of shape and size of gold nanoparticle coated by West Nile virus envelope protein (WNVE) on their immunological responses *in vitro* and *in vivo.*[33] It is showed that the Au rods were most efficiently internalized into cells and induced the secretion of the inflammasome-related cytokines including IL-1β and IL-18. While *in vivo* study showed that Sphere40 (40 nm) was more effective than other shapes (cube and rod) or smaller sphere (20 nm) in antibody production, possibly caused by the production of inflammatory cytokines by Sphere40. However, the detailed mechanisms and generality of the dominant factor underlying the effects of size and shape of Au NPs on immune responses need further investigation. Additionally, although the gold nanoparticles are generally considered safe, they are not biodegradable and repeated use will lead to bioaccumulation, which may lead to long term effects that are yet to be determined.

3.1.3. Silver Nanoparticles—Due to their unique antimicrobial properties, silver nanoparticles are widely used in commercial products and more than 30% of nanomaterial-based consumer products contain nano-sliver.[51] Beyond these widely accepted commercial applications, Xu *et al.* evaluated the adjuvant effect of silver (Ag) nanoparticles (spherical, 141 nm) and showed that AgNPs could induce the increase of OVA specific IgG_1/IgG_{2a} ratio and IgE, indicating the elicitation of Th2-biased immune responses. Further mechanistic study showed that the adjuvant effect of AgNPs was mainly ascribed to the recruitment and activation of local leukocytes, especially macrophages.[34] However, the weakness of AgNPs is the dissolution of the particles that may lead to complete degradation.[51] In addition, AgNPs have been shown to be toxic to mammalian cells and they can also induce acute inflammation in animal lungs and systemic inflammation, so the potential toxicity could outweigh their beneficial adjuvant effects.[51, 52]

3.2.4. 3D Mesoporous Silica Rod—Mesoporous silica nanoparticles have been experiencing an outstanding growth in recent years because of their biocompatibility and unique properties.[53,54] Recently, Kim *et al.* has demonstrated that injected high-aspect-ratio mesoporous silica rods (MSRs) could spontaneously assemble *in vivo* to form macroporous structures that provide a three dimensional cellular microenvironment for host immune cells.[35] In mice, substantial numbers of dendritic cells are recruited to the pores between the scaffold rods. The recruitment of dendritic cells and their subsequent homing to lymph nodes can be modulated by sustained release of inflammatory signals and adjuvants from the scaffold. Moreover, injection of an MSR-based vaccine formulation enhances systemic helper T cells, serum antibody and cytotoxic T-cell levels compared to bolus controls. At the site of the injection, the MSRs are biodegradable within a few months. These findings suggest that plantable MSRs may serve as a multifunctional vaccine platform to modulate

host immune cell function and provoke adaptive immune responses. A follow-up study by Li *et al.* showed that functionalization of MSR scaffold could change their immunogenic potential *in vivo.* MSR scaffold with poly(ethylene-glycol) (PEG) would reduce its immunogenicity and thus decrease immune cell infiltration. In contrast, modifying the MSR scaffold with PEG-Arg-Gly-Asp (PEG-RGD) would enhance immune cell adhesion and infiltration.[36]

In summary, metal and metal oxides represent a group of materials with tunable physicochemical properties that exhibit superior adjuvant potentials. However, there are some potential problems need to be considered during engineered design. First, the biopersistence of the materials, *e.g.,* solubility, clearance by macrophages and dendritic cells, and the site of injection determine material's adjuvancy as well as adverse inflammatory reaction. Thus, material safety is always a major concern, *e.g.,* silver nanoparticles. The injection of metal- or metal oxide-based adjuvants could induce local inflammatory reaction, which can be associated with clinical symptoms of pain, swelling, and redness at the injection site, although these localized reactions are usually mild and of short duration.[44]

3.2. Polymeric Nanoparticles

Polymers represent a type of materials with facile synthesis, superior biocompatibility and biodegradability. Various polymeric nanoparticles have been developed for biomedical application including gene and drug deliveries.[55–61] Synthetic polymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period of days to several weeks.[37]

3.2.1. Poly(lactic-co-glycolic acid)—Among various polymeric nanomaterials, poly(lactic-co-glycolic acid) (PLGA) is a copolymer that has been approved by the Food and Drug Administration (FDA) and widely used in biomedical applications.[55] Desai *et al.* has demonstrated that PLGA nanoparticles containing encapsulated staphylococcal enterotoxin B toxoid showed strong adjuvant properties.[37] In this study, biodegradable nanospheres in the range of 100–150 nm were formulated using PLGA (50:50). Immunization of animals with equal doses of toxoid, either using nanospheres or alum induced a comparable systemic immune response (IgG, IgM and IgA titers). The systemic immune response of animals injected with nanoparticles was comparable to that obtained following injection of alum. However, it reached a maximum at 7 weeks post-immunization, and then gradually declined with time. A booster dose of toxoid at 19 weeks induced a similar secondary immune response that was higher than the primary immune response.[37] In addition, Cruz *et al.* determined the size effect of PLGA on delivering antigen to human dendritic cells *in vitro.*[62] It is demonstrated that encapsulation of antigen resulted in almost 38% degradation for both NPs and micron-sized particles (MPs) 6 days after particle ingestion by DCs, compared to 94% when nonencapsulated and soluble antigen was used. In contrast to the MPs, which were taken up rather nonspecifically, the NPs effectively targeted human DCs. Consequently, targeted delivery improved antigen presentation of NPs and induced antigen-dependent T cell responses at 10–100 fold lower concentrations than non-targeted NPs. However, the potential shortcoming for PLGA as adjuvant is their short half-life because they are often

rapidly degraded. On the one hand, this could be beneficial because it is relatively safe due to their biodegradability; on the other hand, the immune boosting effects are shorter without long term protection, which may require more boosting injections. Future work is needed to design PLGA-based adjuvants that have optimal balance between the two aspects.

3.2.2. Poly (γ-glutamic acid)—Poly(γ-glutamic acid) (γ-PGA) is a capsular exopolymer produced by bacteria. γ-PGA nanoparticles can be degraded by γ-glutamyl transpeptidase that is widely distributed in the human body. Nanoparticles composed of amphiphilic γ-PGA and hydrophobic amino acids can immobilize proteins, peptides, and chemicals onto their surfaces and/or encapsulate these substances into the particles.[38] Wang *et al.* synthesized nanoparticles composed of g-PGA-graft-PAE by a precipitation and dialysis method. They demonstrated that γ-PGA nanoparticles are effective adjuvants that can support the induction of both HIV-1-specific humoral and cellular immune responses, which are needed for an effective anti-AIDS vaccine.[38] Further mechanistic study showed that the production of inflammatory cytokines from macrophages and maturation of dendritic cells were impaired in MyD88 knockout and TLR4-deficient mice compared with their wild-types, when the cells were stimulated with γ-PGA NPs. The immunization of these KO mice with antigen-carrying γ-PGA NPs also results in diminished induction of antigen-specific cellular immune responses, suggesting the involvement of TLR4 and MyD88 signaling pathways.[11] Similar to PLGA, γ-PGA nanoparticles may also be subjected to rapid clearance that makes it difficult to induce long term immune responses.

3.2.3. Chitosan—Chitosan is a type of naturally occurring polysaccharide. Due to its well recognized biocompatibility, low toxicity and degradability by human enzymes, it has been used as delivery vehicles for various biomolecules including peptides, proteins, antigens, genes and oligonucleotides.[63, 64] Due to its superior properties, chitosan is also explored as vaccine adjuvant for enhanced immune responses, especially the Th1 response. Mori *et al.* found that when combined with TLR9 agonist CpG, the CpG-chitosan complex could induce NLRP3-dependent antigen specific Th1 and Thl7 responses.[39] Study by Carroll *et al.* showed that when the chitosan was complexed with Hybrid-1, a fusion protein based on immunodominant antigens from *Mycobacterium tuberculosis*, it mediated enhanced antigen specific Th1 and IgG_{2C} responses that were dependent on both enzyme cyclic-di- GMP-AMP synthase (cGAS) and stimulator of IFN genes (STING).[40] Further mechanistic study demonstrated that the chitosan cationic polymer can actually engage the cGAS-STING DNA sensing pathway for the enhancement of cellular immunity.[40] However, limited human studies are performed using chitosan thus far, and there are no chitosan-related products currently available on the market.[64]

3.2.4. Polyethyleneimine—Polyethyleneimine (PEI) is a type of cationic polymer that has been used as gene delivery reagents *in vitro*[65] and DNA vaccine delivery *in vivo.*[66] It is demonstrated that PEI have potent mucosal adjuvant activity for viral subunit glycoprotein antigens. A single intranasal administration of influenza hemagglutinin or herpes simplex virus type-2 (HSV-2) glycoprotein D with PEI elicited robust antibody-mediated protection from an otherwise lethal infection, and was superior to existing experimental mucosal adjuvants.[7] It is found that linear PEI forms had similar potency as a mucosal adjuvant,

cholera toxin subunit B (CTB), whereas branched PEI forms of 750 kD and 25 kD gave titers of antigen-specific mucosal IgA more than tenfold higher than those elicited by CTB. Detailed mechanistic study showed that PEI formed nanoscale complexes with antigen, which were taken up by antigen-presenting cells, promoted dendritic cell trafficking to draining lymph nodes and induced non-proinflammatory cytokine responses. PEI adjuvanticity required release of host double stranded DNA that triggered Irf3-dependent signaling pathway. Potential weakness of PEI is that high molecular PEI is toxic to cells and the mechanism involves plasma membrane damage and/or lysosomal damage by the proton-sponge effects.[67, 68] Another weakness is that PEI with high molecular weight may bind to DNA tightly that could not be released to the cytosol, thus diminishing the effectiveness of DNA vaccines.[67] So PEIs with optimal molecular weight that are safe and effective should be examined for adjuvant purposes.

3.2.5. pH-responsive polymer—pH-responsive, endosomolytic polymer nanoparticles were originally designed for small interfering RNA delivery.[69] There is advantage to use these particles as adjuvant because after cellular uptake (DC), the endosomolytic property allows the rapid release of digested antigen oligopeptides for presentation outside the cells to boost immune responses. Recently, based on this principle, Wilson *et al.* developed micellar nanoparticles that were assembled from amphiphilic diblock copolymers. The particles were composed of an ampholytic core-forming block and a redesigned polycationic corona block doped with thiol-reactive pyridyl disulfide groups to enable dual-delivery of antigens and immunostimulatory CpG oligodeoxynucleotide (CpG ODN) adjuvants.[70] Conjugation of OVA to nanoparticles significantly enhanced antigen cross-presentation *in vitro* relative to free OVA or unconjugated physical mixture of the parent compounds. Subcutaneous vaccination of mice with OVA-nanoparticle conjugates elicited a significantly higher $CD8^+$ T cell response compared to mice vaccinated with free OVA or a physical mixture of the two components. Significantly, immunization with OVA- nanoparticle conjugates complexed with CpG ODN (dual-delivery) enhanced $CD8^+$ T cell responses 7-, 18-, and 8-fold relative to immunization with conjugates, OVA administered with free CpG, or a formulation containing free OVA and CpG complexed to micelles, respectively. Similarly, dual-delivery carriers significantly increased Th1 responses and elicited a balanced IgG_1/IgG_{2c} antibody response. The pH responsiveness is an advantageous design that could be integrated into other adjuvant platforms. Recently, Tran *et al.* developed composition tunable polymer blend particles by mixing PLGA and a pH-responsive polymer (DMAEMA-co-PAA-co-BMA). It was demonstrated that polymer blend particles are able to deliver antigens into both class I and II antigen presentation pathways *in vitro.*[41] By using a mouse and OVA as a model antigen, it is demonstrated that a significantly higher and sustained level of $CD4^+$ and $CD8^+$ T cell responses as well as comparable IgG production are elicited with polymer blend particles than PLGA particles and commercial vaccine adjuvant, Alum.[41] Similar to PLGA, the stability or degradation rate of these polymers should be optimized to allow time to mount and maintain more effective immune responses.

All together, polymeric materials as vaccine adjuvants are capable of enhancing the vaccine potential against infectious diseases as well as cancers. The adjuvant activities of polymers depends on their solubility, molecular weight, degree of branching and conformation of the

polymer.[71] However, polymeric materials also present potential concerns as other materials do. While benefiting from their unique physicochemical properties, it is difficult to make a prediction on an empirical basis which adjuvant will work most effectively with particular antigens.[72] Although most polymers exhibit good biocompatibility, safety is a major concern for cationic polymers. For example, PEI showed superior adjuvanticity,[7] however, high molecular PEI could cause plasma membrane damage and/or lysosomal damage and lead to cytotoxicity.[67, 68]

3.3. Liposome

Liposomes are a type of material with spherical lipid bilayers ranging from 50 nm to 1000 nm in diameter. It serves as delivery vehicles for biomedical applications, including drug delivery, cancer treatment and vaccine.[73] As vaccine adjuvants, various liposome formulations have been investigated, including DOTAP, DC-Chol, DDA, and DOTIM.[42, 74] Although liposome itself has low immunostimulatory effects, it could increase the number of antigen presenting cells to the administration site[75] and further enhance the cellular uptake of antigens.[76] Commonly, various types of liposome is combined with other immunostimulating ligands, *e.g.,* dsRNA, MPLA, CpG DNA, to generate more specialized and directed immune responses against specific diseases.[74] The weakness for liposomes is their stability, and recent studies on liposomes with solid support such as mesoporous nanoparticles showed substantially prolonged stability that may be used for development of vaccine adjuvants.[77]

4. Immune Activation Mechanisms by ENMs

It is known that the properties of engineered nanomaterials (ENMs) play a major role in influencing the immune system shown as above.[78] Thus, understanding the molecular mechanisms of immune activation is critical in rational design of ENMs for optimal and long lasting immuno-potentiating effects. Although nanomaterials themselves do not have specific immunostimulatory effect, they play an important role in directing immunity towards either a bacterial or viral defense pathway.[79] Currently, the immunostimulatory activity of nanomaterials as adjuvants has been attributed to the following mechanisms: [13, 80–82](1) depot effect that promotes the persistence, stability, and gradual release of antigens;[83, 84] (2) activation of NLRP3 inflammasome;[22, 31] (3) perturbation of dendritic cell membrane;[85] (4) autophagic regulation; (5) delivery of antigens to the draining lymph nodes;[86] (6) Toll-like receptor (TLR)-dependent signal transduction;[11, 87] (7) repetitive antigen display in which the spatial organization of the antigens on the particle surface facilitates B cell receptor co-aggregation, triggering and activation;[88] (8) T cell differentiation;[89, 90] (9) antigen presentation in which exogenously acquired-antigens are processed into MHC class I or MHC class II pathways;[41, 91] (10) host DNA release;[92] and (11) release of soluble mediators such as cytokines, chemokines and immunomodulatory molecules that regulate the immune response (Figure 2).[13]

4.1. Depot effect

Adjuvants can act as depot for antigens, presenting the antigen over a long period of time and maximizing the immune response before the clearance of the antigen.[83, 84] In 1931,

Glenny *et al.* suggested that precipitation of antigens with aluminum salts reduced the rate of antigen elimination from the injection site.[84] In the experiment, the injection sites were collected from guinea pigs 3 days after injection of alum-precipitated or soluble diphtheria toxoid. Then the injection sites were macerated and injected in naive guinea pigs. The recipients of the material from the aluminum-precipitated diphtheria toxoid injected animal developed immune response, but not other guinea pigs. Additionally, it is demonstrated that antigens adsorbed to cationic liposomes compared to neutral liposome -adsorbed antigens have better retention at the injection site.[83] The longer retention time resulted in increased antigen presentation by antigen presenting cells, IFN-γ and IL-17 production, and higher differentiated population of antigen specific T-cells. The cationic liposomes are retained for a longer period of time than neutral liposomes at the site of injection.[83] However, the depot effect was challenged by Holt *et al.* who showed that excision of the injection site after 7 or more days post injection of the aluminum- precipitated diphtheria toxoid, did not interfere with the development of a humoral immune response to diphtheria toxoid.[93]

4.2 NLRP3 inflammasome activation

The NLRP3 inflammasome is an intracellular protein complex that is assembled and activated upon various stimuli in macrophages and DCs.[22] Recently, it was shown that aluminum salts (Alum) could activate the NLRP3 inflammasome and IL-1β production in macrophages, which could explain its ability to induce local inflammation, recruitment of APCs, enhanced antigen uptake, dendritic cell maturation, and stimulation of T-cell activation and T-cell differentiation.[22, 89, 94–100] It is noted that long aspect ratio (LAR) ENMs (*e.g.,* nanowires and carbon nanotubes) trigger activation of the inflammasome secondary to shape-dependent and oxidative stress effects at lysosomal level.[24, 26, 101] Using NLRP3 knockout mice, Eisenbarth *et al.* showed that Alum failed to boost OVA- specific antibody responses in NLRP3, ASC, and caspase-1 knockout mice.[89] Similarly, Kool *et al.* showed that the collection of Alum-induced inflammatory cells in the peritoneal cavity is decreased in NLRP3 deficient mice, supporting the role of the NLRP3 inflammasome in the induction of adjuvant effects.[102] Sun *et al.* shows excellent correlation between NLRP3 activation at cellular level and the generation of *in vivo* adjuvant effects.[31] Although it is generally agreed that Alum is capable of inducing NLRP3 inflammasome activation at the cellular level, there is some disagreement about the necessity of this pro-inflammatory response pathway in generating adjuvant effects *in vivo.*[89, 102–105] Franchi *et al.* showed that the NLRP3 inflammasome was not required for the induction of an antigen-specific antibody response during immunization with Alum.[103] Moreover, Kool *et al.* in another study demonstrated that while NLRP3 deficient mice were partially defective at priming antigen-specific T cells, these animals mounted a normal OVA-specific IgG_1 response.[104] Due to the existing conflicting reports, further research is needed to clarify the role of NLRP3 inflammasome in initiation of immunity by adjuvants *in vivo.*

4.3 Perturbation of dendritic cell membrane

Flach *et al.* reported, independent of inflammasome and membrane proteins, Alum could bind to dendritic cell (DC) membrane lipids with substantial force.[85] Although Alum does not have a specific receptor on the DC surface, it can directly engages lipids in the plasma membrane of DCs, leading to lipid sorting similar to monosodium urate (MSU) crystals that

involves the aggregation of immunoreceptor signaling motif (ITAM)- containing receptors, and subsequent spleen tyrosine kinase (Syk)- and phosphoinositide 3-kinase (PI3K)-mediated phagocytic responses.[85] However, Alum does not enter the cells, it instead delivers the soluble antigen across the plasma membrane. DCs engaged by Alum develop a strong affinity for $CD4^+$ T cells. In contrast, another independent study by Mold *et al.* identified intracellular aluminum adjuvant in THP-1 cells,[106] consistent with the study by Sun *et al.*[31] The discrepancy among these studies, especially on the cellular uptake, may come from the type of cell lines chosen, thus further detailed studies are needed to clarify the role of DC membranes in immunostimulation.

4.4. Autophagic regulation

Autophagic regulation plays a role in the maintenance of cellular homeostasis under normal conditions and during cellular stress.[107, 108] In innate immunity, autophagy is responsible for delivering antigens endosomal toll-like receptors[109] and clearance of damaged mitochondria and ROS.[110] It has been identified as a pathway to deliver cytoplasmic and nuclear antigens to MHC class II molecules for presentation to $CD4^+$ T cells.[107] It has also been implicated in MHC class I cross-presentation of tumor antigen and the activation of $CD8^+$ T cells.[107] Recent study by Li *et al.* indicates that alpha- alumina (α-Al_2O_3) nanoparticles are capable of delivering tumor antigens to the autophagosome-related cross-presentation pathway in dendritic cells. As a result, immunization of mice with a-A1203 nanoparticles conjugated to either a model tumor antigen or autophagosomes derived from tumor cells could lead tumor regression.[111] In addition, autophagy also play a role in NLRP3 inflammasome activation, [108] which would impact the adjuvant effects by nanoparticles.

4.5. Lymph node targeting

Lymph nodes are major sites of B cells, T cells and other immune cells. They are important for the proper function of the immune system, acting as filters for foreign particles and cancer cells.[112–114] Reddy *et al.* showed that nanoparticles can be used as a vaccine platform by targeting lymph node-residing dendritic cells *via* interstitial flow and activating these cells.[86] Following intradermal injection, interstitial flow could transport 25 nm nanoparticles efficiently into lymphatic capillaries and their draining lymph nodes, targeting nearly half of the lymph node-residing dendritic cells, whereas 100 nm nanoparticles were only 10% as efficient. Hanson *et al.* demonstrated that Cyclic dinucleotides encapsulated within PEGylated lipid nanoparticles have the potential to target lymph nodes and further promote both strong antigen-specific T cell priming and high antibody titers.[115] Additionally, Liu *et al.* demonstrated that administration of structurally optimized CpG-DNA/peptide vaccines in mice resulted in marked increases in lymph node accumulation, T-cell priming and enhanced anti-tumor efficacy.[116] Thus, lymph nodes targeting should be considered as a factor in adjuvant design.

4.6. Toll-like receptor (TLR) signaling

Toll-like receptors (TLRs) are a class of proteins that play a critical role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors expressed on macrophages and dendritic cells that recognize structurally conserved molecules derived from microbes.[117] Stimulation of various TLRs could induce distinct patterns of gene

expression that leads to the activation of innate immunity and instructs the development of antigen-specific acquired immunity.[117] Thus far, thirteen different TLRs have been identified in humans and mice. Uto *et al.* described that biodegradable nanoparticles (NPs) elaborated with poly(γ-glutamic acid) (γ-PGA) are able to induce potent innate and adaptive immune responses through TLR4 signaling pathway.[11] The production of inflammatory cytokines from macrophages and the maturation of dendritic cells were impaired in TLR4-deficient mice compared with their wild-types, when the cells were stimulated with γ-PGA NPs. Chen *et al.* demonstrated that the size of nanoparticles that carries the CpG oligodeoxynucleotides (ODN) plays an important role in TLR9 activation.[118] The size of materials affects their ability to regulate endosomal pH, thus the TLR9-mediated differential cytokine productions that regulate both innate and adaptive immunity.[118] In short, selection of optimal TLR agonists and combine that with nanoparticles could enhance the specific adjuvant effects.

4.7. B cell activation

B cells are a type of lymphocyte in the development of humoral immunity. The primary functions of B cells are to synthesize antibodies in response to antigens, to perform the role of antigen-presenting cells and to develop memory B cells after activation by antigen interaction. B cells could also release cytokines that are used for regulating immune functions.[119] Study shows that antigen binding to the B cell receptor (BCR) could induce receptor clustering, cell spreading, and the formation of signaling microclusters, triggering B cell activation.[120] Temchura *et al.* demonstrated that calcium phosphate nanoparticles could be preferentially bound and internalized by antigen-specific B- cells.[121] Co-cultivation of antigen-specific B-cells with the Hen Egg Lysozyme functionalized calcium phosphate nanoparticles also increases surface expression of B- cell activation markers. Further, functionalized nanoparticles are able to effectively cross-link B-cell receptors at the surface of antigen-matched B-cells and were 100-fold more efficient in the activation of B-cells than soluble antigens.[121]

4.8 T cell differentiation

T cells are another type of lymphocyte that plays an important role in cell-mediated immunity. The protective effects of vaccine depend on both the quantity and quality of memory T cells.[122] Studies have demonstrated that the most effective activators of T cells are mature dendritic cells.[122] The T cell-dendritic cell interaction usually requires three signals.[123] The first signal is provided by processed antigenic peptides bound to MHC molecules recognized by the T cell receptors (TCRs), and the second signal by the binding of costimulatory molecules to their ligands on the T cells as well as the third signal that is provided by cytokines and instructs the differentiation of T cells into Th1 or Th2 effector cells. Sokolovska *et al.* found that aluminum-containing adjuvants activate DCs and influence their ability to direct Th1 and Th2 responses.[90] It is demonstrated that aluminum adjuvants could directly enhance the antigen presentation to T cells, the expression of costimulatory molecule CD86 and the production of IL-1β and IL-18 by dendritic cells.[90] These results demonstrate that aluminum-containing adjuvants are not just delivery vehicles for antigens, but directly activate DCs to effectively initiate immune responses and influence the ability of DCs to direct Th1 and Th2 responses.

4.9. Antigen presentation

Dendritic cells (DC) are highly specialized antigen presenting cells that could take up exogenous material from the extracellular environment for presentation in the context of MHC molecules, including MHC I and MHC II. Among these, MHC II molecules are primarily express by professional antigen presenting cells, e.g., dendritic cells, macrophages and B cells.[124] Cytosolic endogenous proteins can be presented by MHC class II molecules. McKee *et al.* showed that aluminum salts could introduce host DNA into the cytoplasm of dendritic cells, where it engages receptors that promote MHC class II presentation and better dendritic cell-T cell interactions.[92] In addition to presentation of antigenic epitopes on MHC class II molecules to $CD4^+$ T cells, DCs can also shuttle antigen to the MHC class I processing pathway for $CD8^+$ T cell activation, a process termed cross-presentation.[125] This enables DCs that have engulfed tumor antigen to activate antigen-specific $CD8^+$ T cells capable of tumor cell killing. Cross presentation present antigens without using the endogenous proteasomal processing pathway used by the MHC II molecule processing. Schnurr *et al.* showed that antigen formulation determines antigen processing.[91] It is demonstrated that ISCOMATRIX adjuvant (a particulate adjuvant comprising saponin, cholesterol and phospholipid) induces efficient cross-presentation of tumor antigen by dendritic cells *via* rapid cytosolic antigen delivery and processing *via* tripeptidyl peptidase II.

4.10. Host DNA release

Either non-self nucleic acids from invading microbes or self nucleic acids left by incomplete clearance during cell damage have been shown to evoke innate immune responses.[126, 127] Those nucleic acids can engage intracellular DNA sensors, including toll-like receptor 9 (TLR9),[128, 129] DNA-dependent activator of interferon-regulatory factors (DAI)[130, 131] and AIM2 inflammasome.[132, 133] The engagement of DNA sensors initiates a cascade of signaling pathways, resulting in secretions of proinflammatory and inflammatory cytokines. Sun *et al.* showed that DNA delivered by hydroxylapatite biominerals activated DAI and AIM2 inflammasomes and mediated production of pro- inflammatory cytokines.[134] In addition, after injection, host DNA could rapidly coat injected adjuvants. McKee *et al.* found that Alum could act as an adjuvant by introducing host DNA into the cytoplasm of antigen-bearing dendritic cells, where it engages receptors that promote MHC class II presentation and stronger DC-T cell interactions.[92] On the same note, DNase coinjection could reduce $CD4^+$ T cell priming.

4.11. Soluble mediators

Soluble mediators such as cytokine, chemokine and immunomodulatory molecules could influence innate and adaptive immune function and T cell polarization.[135] For example, IL-1β is a pro-inflammatory cytokine that has potentiating effects on function of many innate and specific immunocompetent cells and may mediate inflammatory diseases by initiating immune and inflammatory responses.[136–139] Ben-Sasson *et al.* showed that IL-1β could cause a marked increase in the degree of expansion of naive and memory $CD4^+$ T cells in response to antigen challenge, it also increases the proportion of cytokine- producing transgenic $CD4^+$ T cells, especially IL-17- and IL-4-producing cells, strikingly increases

serum IgE levels and serum IgG_1 levels.[140] This study indicates that IL-1β signaling in T cells markedly induces robust and durable primary and secondary $CD4^+$ T cell responses. Another example is IL-10. IL-10 is a cytokine secreted by T_{reg} cells that are associated with immune suppression, and it is considered as a "cytokines synthesis inhibitor factor".[141] It has been shown that DC-based vaccines that induce suppression of IL-10-producting T_{reg} exhibited enhanced efficacy,[142] and inhibition of IL-10 could enhance the magnitude of $CD4^+$ T cell immunity and protection following vaccination.[143]

In summary, various mechanisms are involved in the immunostimulatory activity of nanoscale materials. An effective adjuvant will likely engage multiple pathways and there is no one-size-fits-all model can be derived. Understanding of the detailed mechanisms of these processes could significantly advance the progress of nanomaterial-based immunotherapy for both infectious diseases and cancers.

5. Challenges in Development of ENM-based Adjuvants

The development of nanomaterial-based vaccine adjuvants is faced with several challenges. Though ENM-based adjuvants have been shown to be effective in potentiating immune responses, the physicochemical properties that make them attractive could also potentially generate toxicity, so safety is a major concern.[144] In addition, the exact molecular and immunological mechanisms involved in the adjuvant potentiation remain to be elucidated, and some experimental results are even contradictory, *e.g.,* the involvement of NLRP3 inflammasomes and perturbation of dendritic cell membrane in Alum-induced adjuvant effects. Furthermore, the ENM formulation that works for one antigen may not work for another because of the differential immune potentiating mechanisms. Thus, further work needs to be done to design safer and effective ENM- based adjuvants for specific antigens.

In addition, cancers are still one of the leading causes of morbidity and mortality worldwide. In 2012, there are 14 million new cases and 8.2 million cancer-related deaths, and the new cases are predicted to rise by 70% over the next two decades.[145] Thus, effective preventative and therapeutic treatment are highly in demand. Beyond traditional treatments including surgery, chemotherapy, and radiation therapy, vaccination for cancer prevention and treatment have been drawing more and more attentions. Firstly, vaccination can prevent diseases that lead to cancers. For example, the hepatitis B vaccine is 95% effective in preventing the development of chronic disease and liver cancer;[5] Gardasil, a human papillomavirus (HPV) vaccine, can protect against two types of HPV that cause 70% of cervical cancer, 70% of vaginal cancer and 50% of vulvar cancer.[6] Furthermore, vaccination can be used for cancer treatment. Thus far, the US FDA has approved immunotherapies including sipuleucel-T (Provenge) for metastatic prostate cancer, talimogene laherparepvec (T-VEC) for metastatic melanoma,[146] and checkpoint inhibitors targeting PD-1 (Tecentriq) and CTLA-4 (Yervoy) for bladder cancer and metastatic melanoma, respectively.[147, 148] Among these immunotherapies, cytokines and growth factors, *e.g.,* interleukin 2 (IL-2), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are commonly used as adjuvants in vaccines to augment the immune response.[146] Currently, with the advancement of nanotechnology, nanomaterials with immunostimulatory properties are either served as adjuvants or carrier to deliver adjuvants or checkpoint inhibitors to augment the immune

responses targeting cancer cells.[111, 149, 150] Although such studies are in pre-mature stage, many promising nanomaterials are under pre-clinical and clinical investigations.[151, 152] As we're gaining more understanding on the acting mechanisms of nanomaterial-based adjuvants, therapeutic cancer vaccines with nano scale adjuvants could be developed to benefit the cancer patients.

6. Conclusions and Perspectives

The usage of engineered nanomaterials that possess unique physicochemical properties in adjuvants enables researchers to potentially achieve improved protection against infectious diseases. Currently, there are many nanomaterial-based adjuvants available or in development that have been shown to be effective to potentiate immune responses induced by a variety of antigens. However, there are some challenges on safety and understanding of the molecular mechanisms, which need to be addressed. Through a systemic approach including engineered design to control the properties and further exploration on the mechanisms of action using *in vitro* and *in vivo* approaches will answer these challenges to develop better ENM based adjuvants.

A new trend on adjuvant design is also emerging that could be beneficial for the development of ENM-based adjuvants. Recent studies show that when a combination of nanomaterial adjuvants and additional specific immunostimulatory substances, *e.g.,* TLR agonists, they can specifically activate Th1 and/or Th2 responses. For example, triggering of TLR4, 7 could stimulate the production of cytokines/chemokines (TNF-α, IL-2, IL-12 and IL-6) and type **I** interferons (IFNs) that promote the immune system to activate Th1 responses to eliminate exogenous influenza viruses.[153] Clinical trials suggest that TLR ligands can be safe and effective as vaccine adjuvants, and there are vaccines already licensed in the US and Europe containing such ligands.[154] Combinational adjuvant, such as AS04, is able to elicit multiple protective immune responses. Thus, combining nanomaterial-based vaccine adjuvants with these novel TLR ligands that modulate the innate immunity could generate synergistic effects to significantly benefit development of more effective vaccine adjuvants. At the same time, this may reduce the amount of antigen and/or the number of immunizations needed. These efforts will facilitate the development of safe and more effective nanomaterial-based adjuvants to combat infectious diseases and cancer. Additionally, ENM-based adjuvants could be helpful for the development of therapeutic vaccines for cancers as well as diagnostic purpose for autoimmune diseases, which are under intense research and investigation.[155–158]

Supplementary Material

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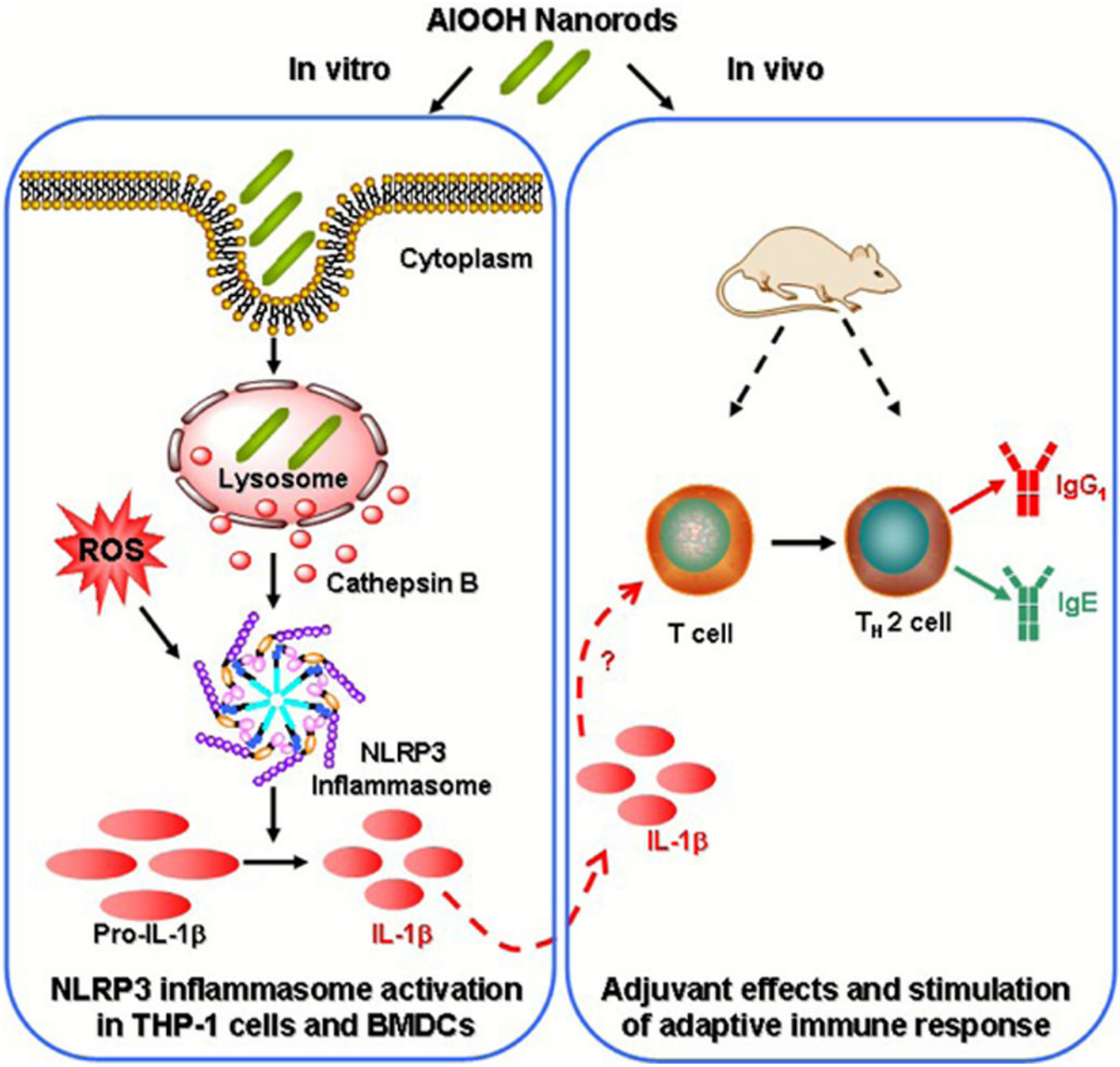

Figure 1.

Engineered aluminum oxyhydroxide (A100H) nanorods as vaccine adjuvants. The shape, crystallinity and hydroxyl content play an important role in NLRP3 inflammasome activation and boosting of antigen-specific immune responses.

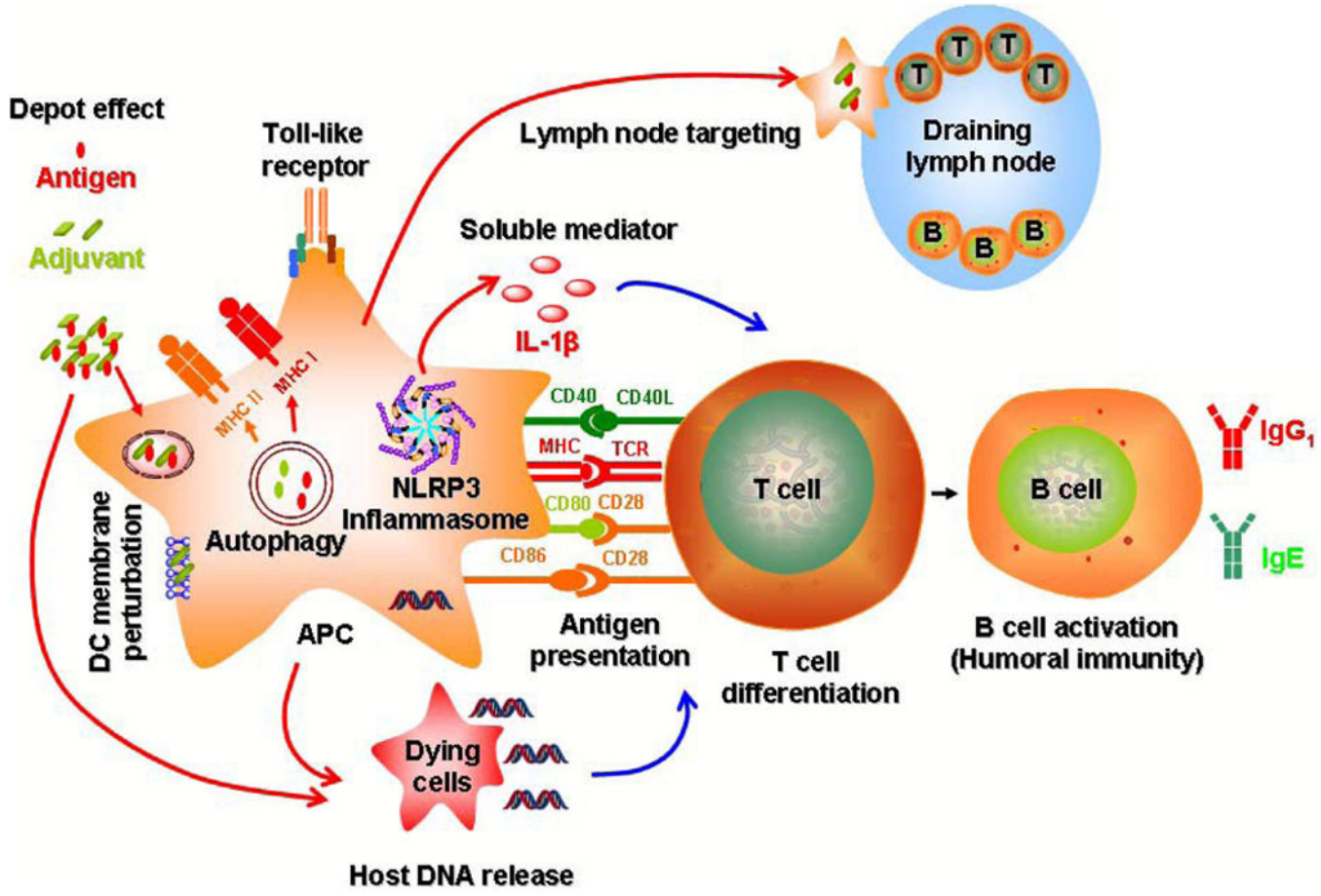

Figure 2.

Mechanisms of immune system activation by engineered nanomaterials include (1) depot effect; (2) activation of NLRP3 inflammasome; (3) perturbation of dendritic cell membrane; (4) autophagic regulation; (5) delivery of antigens to the draining lymph nodes; (6) Toll-like receptor (TLR)-dependent signal transduction; (7) B cell activation; (8) T cell differentiation; (9) antigen presentation; (10) host DNA release; and (11) release of soluble mediators.

Table 1.

Approved vaccine adjuvants in human and their active components.

Adjuvants	Components	Disease	Stage	Reference
Aluminum salts	Aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate, aluminum hydroxyphosphate sulfate	Hepatitis A, hepatitis B, DTaP-HepB- IPV, human papillomavirus etc.	Licensed (US, EU)	[4, 9]
AS03*	Squalene, DL-α-tocopherol, polysorbate 80	Influenza	Licensed (US, EU)	[4, 9]
AS04	Aluminum-absorbed TLR4 agonist	Hepatitis B, human papilloma virus	Licensed (US, EU)	[4, 9]
MF59	Oil-in-water emulsion of squalene oil	Influenza	Licensed (US, EU)	[4, 9]
Virosome	Viral envelop	Influenza and Hepatitis A	Licensed (EU)	[10]

*AS03 is included in the H5N1 influenza vaccine, however, is not commercially available.

Table 2.

Nanomaterial-based vaccine adjuvants.

Nanomaterials	Physicochemical Properties in Study	Model Antigens	Reference
Aluminum hydroxide	Size	*Bacillus anthracis* protective antigen	32
Aluminum oxyhydroxide	Shape, crystallinity, hydroxyl content	OVA	31
Gold	Shape, size	West Nile virus envelope protein	33
Silver	N/A	OVA	34
Mesoporous silica	3-D rod and surface chemistry	OVA, CpG-ODN	35, 36
PLGA	N/A	Staphylococcal enterotoxin B toxoid	37
γ-PGA	N/A	HIV-1	38
Chitosan	N/A	OVA, Hybrid-1	39, 40
PLGA-DMAEMA-co- PAA-co-BMA	Blend ratio	OVA	41
PEI	N/A	Influenza hemagglutinin, HSV-2 glycoprotein D	7
DDA liposome	N/A	BBG2Na, a recombinant fusion protein produced in *E. coli*	42

Table 3

Aluminum salts in licensed vaccines.[43–47]

Vaccine	Trade name and manufacture	Age of usage	Type of aluminum salts
Anthrax	Biothrax, Emergent BioSolutions, Inc.	18–65 (y)	Aluminum hydroxide
Td	Decavac, Sanoft Pastern Limited	>7(y)	Aluminum potassium sulfate
Td	Tenivac, Sanoft Pasteur Limited	>7(y)	Aluminum phosphate
DTaP	Daptacel, Sanofi Pasteur Limited	6 (w)-6 (y)	Aluminum phosphate
Tdap	Adacel, Sanofi Pasteur Limited	10–64 (y)	Aluminum phosphate
Tdap	Boostrix, GlaxoSmithKline Biologicals	>10 (y)	Aluminum hydroxide
DTaP-IPV	Kinrix, GlaxoSmithKline Biologicals	4–6 (y)	Aluminum hydroxide
DTaP-HepB-IPV	Pediarix, GlaxoSmithKline Biologicals	4–6 (y)	Aluminum hydroxide
DTaP-IPV/Hib	Pentacel, Sanofi Pasteur Limited	6 (w)-4 (y)	Aluminum phosphate
Haemophilus influenzae	PedvaxHIB, Merck & Co., Inc	2–71 (m)	Aluminum hydroxyphosphate sulfate
Haemophilus influenzae/Hepatitis B	Comvax, Merck & Co., Inc	2–15 (m)	Aluminum hydroxyphosphate sulfate
Hepatitis A	Havrix, Glaxo SmithKline Biologicals	>12 (m)	Aluminum hydroxide
Hepatitis B	Engerix-B, GlaxoSmithKline Biologicals	All age	Aluminum hydroxide
Hepatitis B	Recombivax HB, Merck & Co., Inc	All age	Aluminum hydroxyphosphate sulfate
Hepatitis A / Hepatitis B	Twinrix, GlaxoSmithKline Biologicals	>18 (y)	Aluminum hydroxide, Aluminum phosphate
Human Papillomavirus (HPV)	Cervarix, GlaxoSmithKline Biologicals	9–25 (y)	Aluminum hydroxide
Human Papillomavirus (HPV)	Gardasil, Merck & Co., Inc Ixiaro Intercell	9–25 (y)	Aluminum hydroxyphosphate sulfate
Japanese Encephalitis	Biomedical Ltd. PCV13-Prevnar 13,	>2 (m)	Aluminum hydroxide
Pneumococcal	Wyeth Pharmaceuticals, Inc.	>65 (y)	Aluminum phosphate

AP: acellular pertussis; D: diphtheria; Hib: Haemophilus influenzae type b; IPV: inactivated poliovirus; T: tetanus; w: weeks; m: months; y: years.