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REVIEW

Tolerogenic Nano-/Microparticle Vaccines for Immunotherapy

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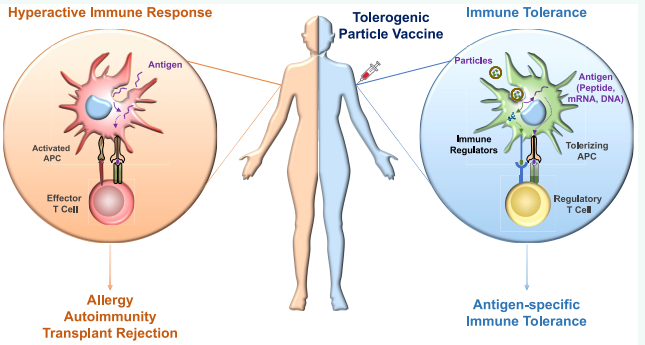
ABSTRACT: Autoimmune diseases, allergies, transplant rejections, generation of antidrug antibodies, and chronic inflammatory diseases have impacted a large group of people across the globe. Conventional treatments and therapies often use systemic or broad immunosuppression with serious efficacy and safety issues. Tolerogenic vaccines represent a concept that has been extended from their traditional immune-modulating function to induction of antigen-specific tolerance through the generation of regulatory T cells. Without impairing immune homeostasis, tolerogenic vaccines dampen inflammation and induce tolerogenic regulation. However, achieving the desired potency of tolerogenic vaccines as preventive and therapeutic modalities calls for precise manipulation of the immune microenvironment and control over the tolerogenic responses against the autoantigens, allergens, and/or alloantigens. Engineered nano-/microparticles possess desirable design features that can bolster targeted immune regulation and enhance the induction of antigen-specific tolerance. Thus, particle-based tolerogenic vaccines hold great promise in clinical translation for future treatment of aforementioned immune disorders. In this review, we highlight the main strategies to employ particles as exciting tolerogenic vaccines, with a focus on the particles' role in facilitating the induction of antigen-specific tolerance. We describe the particle design features that facilitate their usage and discuss the challenges and opportunities for designing next-generation particle-based tolerogenic vaccines with robust efficacy to promote antigen-specific tolerance for immunotherapy.



KEYWORDS: *Tolerogenic vaccines, nanoparticles, microparticles, regulatory T cells, immune response, autoimmune diseases, antigen-specific tolerance, vaccine delivery strategy*

1. INTRODUCTION

Immune tolerance is a highly regulated process to restrain the generated immune responses against self-antigens or environmental innocuous antigens. This functional unresponsiveness against antigens from self or the environment is maintained under normal physiological conditions to prevent immune response against healthy tissues and normal cells. To maintain the immune tolerant state, the body has employed both central and peripheral tolerance mechanisms. Central tolerance represents the known negative selection processes eliminating the self-reactive or autoreactive T cells in the thymus and bone marrow.[1,2] Peripheral tolerance inhibits activated T cells in peripheral tissues and secondary lymphoid organs. By employing multiple mechanisms, peripheral tolerance could impede the expansion of self-reactive/autoreactive T cells and restore immune homeostasis.[3] However, deficit or breakdown of tolerance can lead to allergic disorders and/or cause autoimmune diseases, conditions in which the immune system cannot differentiate the host tissues/cells and the normal non-self-substances and then induce aberrant activation of T and B cells and trigger inflammatory cytokine secretion and antibody production causing tissue injury. The worldwide incidence of these abnormal immune conditions is accelerating, including the currently most prevalent autoimmune diseases, such as multiple sclerosis (MS), type 1 diabetes (T1D), rheumatoid arthritis, and lupus. Other conditions led by a lack of immune tolerance are hypersensitive allergic responses against various nonharmful foreign proteins (known as allergens) that manifest clinically as food allergy, allergic asthma, allergic rhinitis, ocular allergy, and allergic skin inflammation. Allergic asthma and rhinitis are local allergic inflammation, while

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anaphylaxis is systemic and generally driven by IgE antibodies. Other areas where unwanted immune reactions are generated, especially raising the production of neutralizing antibodies against repeatedly delivered viral vectors used for gene therapy or recombinant protein drugs, could lead to loss of therapeutic effects.[4,5] The field of transplantation still calls for treatments including tolerance induction for the recipients to the donors, protecting the allogenic transplants from attack and destruction or vice versa.[6] Current estimates suggest that ∼10% of the global population suffers from one of immune disorder condition or autoimmune disease.[7,8] Various investigations predict a worldwide rising trend in multiple autoimmune diseases, and the yearly increases in the globe incidence and prevalence are estimated to be ∼19.1% and ∼12.5%, respectively.[9,10]

Traditional treatments of these diseases often involve the systemic administration of immune suppressants, including standard anti-inflammatory agents such as small molecules and corticosteroids, to inhibit broad inflammation and postpone the destruction to healthy cells and tissues.[11] More targeted remedies include the use of recombinant cytokines and monoclonal antibodies (mAbs), for instance, adalimumab (cytokine depletion antibody) and rituximab (B cell depletion antibody).[12−14] These treatments are more specific compared to the conventional ones, but they do not have selectivity since they bind their target receptors displayed on all healthy and abnormal cells.[15] Therefore, they could cause harmful effects to normal cell function.[12,15,16] The cell-based strategies, which include adoptive immunotherapy and stem cell transplantation to inhibit autoreactive T cells and restore immune tolerance, face manufacturing, purification, and scale-up challenges to realize their full potential.[17,18] Moreover, current therapies often require lifelong, repeated, and even daily administration and are not curative.[19,20] Besides, the induced tolerance usually has insufficient coverage and requires the periphery regulation provided by specific antigen-presenting cells (APCs) on T cells.[21,22] Altogether, these drawbacks of existing treatments highlight the importance of engineering safer, more effective, and long-term approaches inducing more selective immune tolerance.

Tolerogenic vaccines, also known as “inverse vaccines”, represent significant approaches to invoke and maintain antigen-specific tolerance-based immunotherapy.[23,24] Mainly through the differentiation and expansion of regulatory T cells (Tregs), tolerogenic vaccines could achieve long-lasting effects on inhibiting pathological immune response to allergens or autoantigens.[25,26] Besides, tolerogenic vaccines could induce the apoptosis and anergy of autoreactive T cells by passive or deletional tolerance.[27] Tolerogenic vaccines have been inspired by allergen-specific immunotherapy (AIT).[28,29] The mechanisms of initiating and maintaining antigen-specific tolerance are extremely complex integrations and cascades of multiple signals.[30] The types of signals, and the context of where and when the signals are encountered during the generation of the ensuing immune response, define the specific features.[30,31] How can tolerogenic vaccines selectively function with Helper T cells (Th cells) instead of riskily activating cytotoxic T cells and B cells to initiate inflammatory responses? In a similar fashion to conventional vaccines, the induction of dominant tolerance by the tolerogenic vaccines can be briefly explicated by the “three-signal” model of T-cell activation.[32] All delivered autoantigens, alloantigens, or allergens would be processed into peptides, which then bind major histocompatibility complex (MHC) class II proteins and display on the APC surface to present to Th cells.[33] This process providing an antigenic trigger to T cells is referred to as signal 1. Then tolerogenic vaccines would lead to the maturation of APCs and up-regulate co-inhibitory molecule expression by both APCs and T cells, for instance, cytotoxic T-lymphocyte−associated antigen 4 (CTLA-4), programmed death 1 (PD-1), and inducible T-cell co-stimulator (ICOS), and secrete anti-inflammatory cytokines and chemokines to provide the second and third signal for Th cell expansion. Tolerogenic vaccines focus on the tolerogenic APCs, e.g., dendritic cells (DCs), liver sinusoidal endothelial cells (LSECs), or, alternatively, activated M2 macrophages, facilitating their encounter with Th cells in such a tolerogenic environment. Then T cells become “anergized” with compromised effector functions, leading to differentiation into regulatory T cells (Tregs) that induce functional unresponsiveness of antigen/allergen-specific T or B cells.[34] The aforementioned AIT, as the earliest tolerogenic vaccine, which involves repeated sublingual application or subcutaneous (sc) injection of the sensitizing allergen, is the earliest clinically effective treatment for the induction of immune tolerance to specific allergens. There are unforeseeable possibilities for adverse events as well as serious anaphylaxis. Additionally, these therapies usually need long-term, even lifelong, treatment to prevent regaining reactivity after cessation.[20] Thus, an urgent need remains for developing a tolerogenic vaccine to serve as long-term antigen-specific immunotherapy.

Emerging biomaterials could serve as applicable tools for the design of tolerogenic vaccines to induce and maintain antigen-specific immune tolerance.[35] A few biomaterial platforms have been applied as antigen delivery and adjuvant systems in conventional vaccines stimulating immune responses, exhibiting features to activate and maturate APCs, possessing antigen controlled release, and instructing the subsequent T cell and B cell responses.[36] In the same manner, the physicochemical forms and characteristics of biomaterials play a role in tolerogenic vaccines, delivering antigens/allergens and impacting the magnitude and quality of the immune response.[21] Commonly employed biomaterial configurations include nanoparticles (NPs), microparticles (MPs), or other self-assembling structures, which can mainly facilitate targeted delivery and controlled release as well as provide co-delivery opportunities for multiple immune signals, and implantable scaffolds, hydrogels, or other bulk materials, which can function as a depot for local delivery of immune signals (including NPs and MPs) and engineer or reverse into a localized tolerogenic microenvironment.[35,37,38] Besides biomaterial platforms, alternative forms of antigens and allergens are being investigated and developed, for instance, custom-designed, precisely predicted, disease-relevant antigen epitopes and peptides, directly interacting with APCs and effectively dampening Th1 and Th2 responses without inducing autoimmune complications.[39] All of these features are catalyzing opportunities for tolerogenic vaccines to bring changes in antigen-specific tolerance induction.

In this review, we present leading approaches in which tolerogenic vaccines are being designed to induce antigen-specific tolerance, promote tolerogenic immune function, and tackle challenges in immune disorders including allergies and autoimmune diseases. We mainly focus on describing strategies harnessing NPs and MPs to deliver antigens or allergens and immunomodulatory agents and discussing strategies for how to

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attune their intrinsic physical forms and chemical properties that promote tolerance in immune cells and examples using improved approaches for particle design. Key references illustrating particle features are summarized in Table 1. Next, we present recent progress and efforts in identification and application of defined antigen/allergen epitopes and other derivatives as well as the use of mRNA and DNA in an attempt to speed up the development and improve clinical efficacy. We conclude by providing perspective and exhibiting efforts for approaches and strategies to achieve optimal particle-based delivery vehicles and antigens, and the potential of combining tolerogenic vaccines with other treatment options including extracellular vesicles (EVs) and chimeric antigen receptors (CARs) is also analyzed.

Table 1. Nano-/Microparticle-Based Tolerogenic Vaccine Approaches to Promote Immune Tolerance

strategy for antigen-specific tolerance induction	refs
Nanoparticles	
passive targeting of tolerance-related organs by manipulating particle sizes	58−60
active targeting of particular tolerogenic APCs (DCs, LSECs) by conjugating specific ligands onto the surface	73, 74, 77, 81
selecting optimal immunization routes to the anatomical location of tolerogenic cells	82, 83
harnessing oral tolerance by delivery of antigen through the oral route	94, 95
targeting autoantigens to tolerogenic zones in spleen	85, 110−114
delivering antigens to liver to involve them in the natural tolerogenic process	124, 128, 129
targeting B cell-specific tolerance	130, 132−135
acting directly on effector T cells and presenting antigen without co-stimulation	139
delivery regulatory cues directly to T cells	147, 148
delivering immunomodulators to DCs, e.g., AHR agonist, rapamycin, NF-κB inhibitor, and TLR agonist, to lock in tolerogenic response	151−154, 156, 163−168, 177−179, 188
Microparticles	
forming immunomodulatory depots to modulate the local microenvironment	189−191
mimicking artificial immune organs to reprogram the immune cells *in situ*	192−194
promoting homeostasis both locally and systemically	197−199

2. NP DELIVERY OF TOLEROGENIC VACCINES TO INDUCE ANTIGEN-SPECIFIC TOLERANCE

The majority of research applying biomaterials to promote antigen-specific tolerance has focused on harnessing NPs (also known as tolerogenic NPs) which have significant advantages over conventional delivery systems, for instance, (1) protection of antigen/allergen from protease degradation, (2) reduction of off-target effects due to adjustable size/shape and surface properties as targeting moieties, (3) co-delivery capacities for multiple therapeutic agents to elevate the immune response, and (4) regulating abilities as versatile compositions and administration routes to control the release of antigen cargo as well as the therapeutic agents.[21,34] Lipids, metals, and synthetic or natural polymers are prominent materials used to promote immune responses.[40] Liposomes are NPs with an amphipathic lipid bilayer membrane and inner aqueous phase.[41,42] Lipid nanoparticles (LNPs) are made of ionizable lipids with positive charge at low pH and neutral charge at physiological pH.[43] In particular, LNPs have been thoroughly investigated and not only obtained huge success as efficient COVID-19 mRNA vaccines but also competently entered the clinic for siRNA and mRNA delivery therapies treating multiple diseases.[43,44] Metal and metal-oxide NPs are more stable and widely applied as delivery systems in tumor immunotherapy; however, bio-persistence and toxicity issues should not be ignored.[45−47] Various natural and synthetic polymers such as poly(lactic-*co*-glycolic acid) (PLGA), poly(lactic acid), polystyrene, chitosan, and acetylated dextran have been used to fabricate NPs.[48−50] Biodegradable polymeric NPs are favored due to their excellent safety profile, higher stability over liposomes, and unique ability to conduct controlled release of antigen and therapeutic

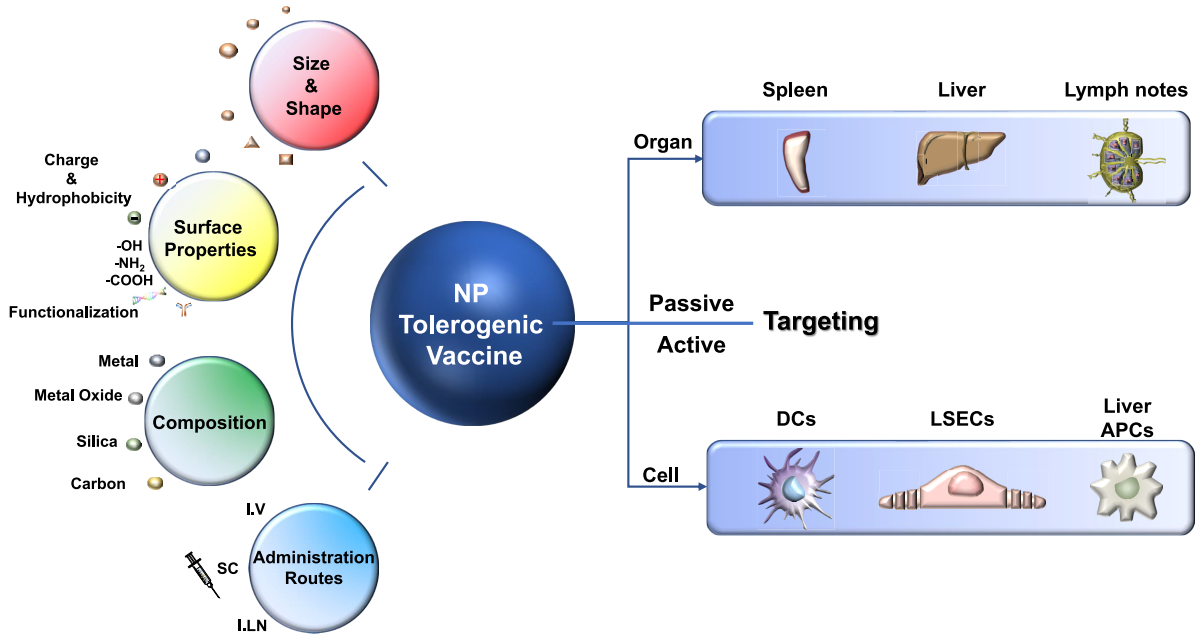

Figure 1. Particle-based tolerogenic vaccines with major physicochemical characteristics, targeting from organ to cell level, could accomplish the goal of precise delivery and tolerance induction.

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payload cargos.[50] Besides the material forming the nanoparticle, several parameters have to be carefully considered when designing tolerogenic NPs, since size and shape as well as surface characteristics remain crucial parameters during NP interaction with immune cells, tissues, and biological fluids.[30,50,51] Motivated by the initial success of nanomedicine for cancer immunotherapy and COVID-19 mRNA vaccines, encouraging approaches to attune the immune system by tolerogenic NPs to treat autoimmune diseases and allergies are being adapted and developed. In this section, the main strategies in which NP-based tolerogenic vaccines can induce antigen-specific tolerogenic responses are listed.

2.1. Targeting Specific Immune Tolerogenic Tissues or Cells to Promote Tolerance. One of the features of tolerogenic NPs is their ability to target encapsulated cargos to the desired specific immune cells or tissues. Targeting abilities, here, not only involve passive drainage or residence to tolerogenic-related organs by mainly managing the NP size, hydrophobicity, and surface charge but also actively targeting payloads to desired cells or tissues by directly modifying NPs with targeting moieties (e.g., antibodies, targeting ligands, etc.).[40,52] Targeting abilities of NPs bring in notable benefits compared with systemic administration of free antigens/allergens by facilitating dose-sparing as well as attenuating exposure of cargo to nontargeted tissues or cells.[53] Rapidly emerging nanotechnologies further enable NPs to target a particular cell population of interest by targeting specific receptors.[54] Targeting moieties and strategies consequently become necessary to precisely control the delivery of antigens/allergens as well as immunomodulatory agents to promote antigen-specific tolerance (Figure 1).

NP delivery could be considered as a passive targeting effect to APCs since they can be located in particular tolerance-related tissues or organs and then internalized and processed by tolerogenic APCs.[55−57] The size of NPs is imperative in dictating the intracellular fate of antigens.[58] NPs with a size under 200 nm can directly diffuse into lymph nodes (LNs) or effectively drain by afferent lymphatics within hours of injection.[21,59,60] Depending on the chosen injection routes (intramuscular, subcutaneous, intradermal, or intravenous) in the experiments, NPs having a diameter from 200 to 500 nm, located in the lung, liver, or spleen, reach the lymph nodes with the help of migratory APCs or remain at the injection site.[21,61,62] NPs under 100 nm can be efficiently taken up by clathrin-mediated endocytosis, and larger NPs are internalized via phagocytosis or micropinocytosis.[63,64] The differences in NP size directly impact their biodistribution within the body and thus affect immune responses ascribed to their interaction with various APC subpopulations.[65] Certain immune cells are favored; targeting ligands and strategies are therefore being applied to improve the efficiency of priming antigen-specific tolerance.

DCs are one of the most important APCs which are pivotal in directing immune responses or tolerance.[66,67] This dual capacity depends on the DC subtype, as well as the stimuli and signals which DCs receive from the local environment, mainly consisting of MHC complex, co-inhibitory/stimulatory molecules, and cytokines.[68] As a result, the use of immunomodulatory agents such as rapamycin, vitamin D3, retinoic acid, or specific cytokines (IL-10, TGF-β) has been key in determining the tolerogenic function of DCs.[69] NP-enabled modulation would be achieved by targeting particular DC subsets with specific surface receptor expression profiles and ensuring antigen/allergen delivery.[21] The currently prevalent application of antigen−antibody conjugates targeting DEC-205 (endocytic c-type lectin) receptor expressed mainly by cDC1s (CD141$^+$ conventional DCs) has shown encouraging results in mouse models of colitis and MS to enhance tolerance.[69−71] DCs also express other c-type lectins, e.g., DC-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN; CD209).[72] Coates et al. explored porous silicon antigen and drug-loaded NPs (pSiNPs) coupled with DC-SIGN monoclonal antibodies on the surface and showed the enriched uptake of NPs by murine myeloid CD4$^+$ and CD8α^+ DC subsets *in vivo*.[73] Also, the increase of Treg production has been observed *in vivo* until 40 days after the injection of both antigen- and rapamycin-loaded pSiNPs.[74]

To delve into the immunosuppressive effects of the liver and its unique system of APCs, LSECs are the primary target cells of interest, which is crucial in inducing immune suppression mainly through the generation of antigen-specific Tregs and TGF-β production.[75,76] Previous work in our lab exploited a versatile biodegradable FDA-approved polymer PLGA nanoparticle platform with a size of ∼200 nm that the LSECs proficiently internalize and coated with ApoB peptide to target the stabilin-1/2 scavenger receptors exclusively expressed on LSECs.[77] The ligand-coated NPs have been proven to target LSECs and enhance antigen delivery *in vivo*. Ovalbumin (OVA)-induced airway allergic inflammation was significantly suppressed with an enhanced level of TGF-β production and the appearance of Foxp3$^+$ Tregs in the lung.[77] These results proved the feasibility of this tolerogenic NP platform for antigen/allergen delivery to LSECs by managing both particle size and surface characteristics. In addition, Hubbell et al.[81] identified the C-type lectin receptors responsible for inducing the tolerogenic process by liver APCs, including liver DCs, Kupffer cells (KCs) and LSECs, and the terminal *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GluNAc) residues on the proteins/peptides binding C-type lectin receptors expedite T cell tolerance induction in the liver.[78−80] They synthesized particles composed of antigen−p(GalNAc)/(GluNAc) conjugates and proved an attenuated effector response along with an enrichment of antigen-specific Treg in an OVA-specific transgenic CD8$^+$ (OTI) and CD4$^+$ (OTII) T cell challenge model.[81]

Besides the surface phenotype, the anatomical location of targeted cell should also be considered.[21] Therefore, the administration route could involve the localization efficiency of targeted NPs. Miller et al. sought to choose the optimal route for delivering NP-based vaccine in a murine model of MS to prevent clinical symptom development of R-EAE (relapsing experimental allergic encephalomyelitis, an autoimmune disorder stimulating MS). Using the same standard dose of antigen-coupled PLGA NPs (average diameter of ∼400−500 nm), the intravenous (iv) administration was significantly more effective than the intraperitoneal (ip) administration, and no protective effects were provided via either subcutaneous (sc) or oral routes.[82,83] This result was also consistent with their previous result that targeting tolerogenic APCs in the spleen and liver mainly depends on iv and ip injection.[84,85] It is therefore essential to design the particle size and surface properties to optimize the immunization routes as well as select pharmaceutical cargos for achieving the ideal tolerogenic responses.

2.2. Altering the Trafficking, Processing, and Presentation of Antigens/Allergens to Promote Immune

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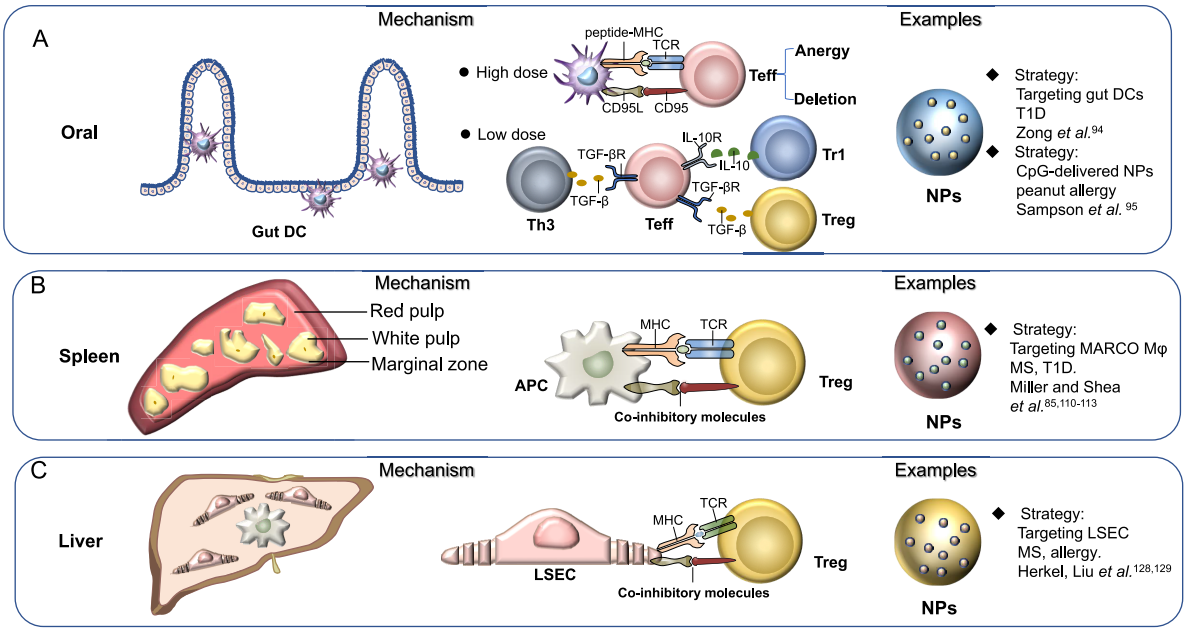

Figure 2. Particles harnessing the natural tolerogenic processes to deliver antigen and facilitate the antigen/allergen processing and presentation to promote immune tolerance. Three main tolerogenic processes are depicted through oral tolerance (A), spleen (B), and liver (C).

Tolerance. To explore NPs as tolerogenic vaccines to prime antigen-specific tolerance, their delivery of antigens/allergens and the following process of NP-delivered antigen trafficking and presentation constitute the primary and foremost signal (signal 1) to effector T cells and B cells. This strategy focuses on harnessing the natural tolerogenic processes for the delivered antigen in the tolerogenic environment to induce antigen-specific tolerance.

Oral tolerance remains active and well-regulated with the distinct intestinal tract maintaining close contact with food antigens, gut flora antigens, and other molecules.[86] There are several possible pathways for antigen internalization in the intestinal epithelium.[87] Under the conditions of oral antigen delivered at high doses, effector T cells could lead to anergy or deletion with the inhibition signal (CD95 and CD95 ligand), respectively.[88,89] At low doses, activation of Tregs, type 1 regulatory T cells (Tr1), and Th3 was initiated, and suppression of effector T cell responses could occur mainly though suppressive cytokines (IL-10 and TGF-β)[88] (Figure 2A). Important tolerogenic mechanisms are established to avoid inflammatory response, and DCs from gut Peyer's patches tend to secrete anti-inflammatory cytokines and induce Tregs.[90,91] Hence the intestinal immune system makes a potentially proper target for tolerance induction. However, in the gastrointestinal tract by oral delivery, antigens are not stable, easily degrade, and are poorly taken up by DCs due to obstruction and adsorption by the intestinal mucus and epithelial cell layer barriers.[92,93] Zong et al. developed a targeted NP delivery system that facilitates targeting of gut DCs involving antigen (H6P, heat shock protein 65-6×P227) in the gut DC-based processing and presentation.[94] The oral vaccination prevented diabetes in mice, and $CD4^+CD25^+FOXP3^+$ Tregs were found to participate in the tolerance induction.[94] Besides the attempt using NPs targeting and delivering antigen to gut DCs, Sampson et al. used CpG-coated PLGA NPs incorporating peanut extract in a murine model of peanut allergy.[95] Mice with NP treatment were protected from anaphylaxis under up to five oral peanut challenges with less change in body core temperature, lower anaphylaxis scores, and lower levels of plasma histamine compared to other groups treated with allergen alone or no treatment.[95] A decrease in peanut-specific IgE and IgG levels was observed with an increase in IgG2a levels indicating Th1 response deviation. Peanut recall responses in splenocytes were also conducted, and the group treated with NPs showed decreased production of Th2 cytokines (IL-4, IL-5, and IL-13) and increased IFN-γ production.[95] Despite the effectiveness in mice, oral tolerance in human clinical trials is widely discouraging. Palforzia, made up of powdered peanut protein extract, remains to be the only oral desensitization therapy to treat peanut allergy approved by FDA.[96] Besides the high cost and up to 6-month treatment period, the possibility of anaphylaxis has not been proven to be excluded, and long-term efficacy has not been accomplished.[96−99] That makes it difficult to depend on harnessing the oral-route tolerance approach only for the nanoparticle-based tolerance induction to translate into clinics.

Studies have been reported focusing on the spleen, the largest secondary lymphoid organ, to reverse its traditional role to prime immune response for the design of tolerogenic vaccines (Figure 2B). The spleen hosts immunological functions in surveying and clearance of abnormal cells, cell debris, and red blood cells.[100] Thus, the spleen is divided by its function into three major zones: the white pulp is the primary immunologic region with T and B cell zones but makes up less than a quarter of whole splenic tissue; the red pulp makes up the majority of the spleen with a scavenging function distinct from white pulp, and the marginal zone between them is a bridge connecting the white and red pulp.[100,101] The immunological function is favored and investigated by researchers to develop vaccines against pathogens and tumors. Meanwhile, the filter and cleaning role of the spleen without activating immune responses attracts the researcher to regard it as a promising opportunity to develop a tolerogenic vaccine by

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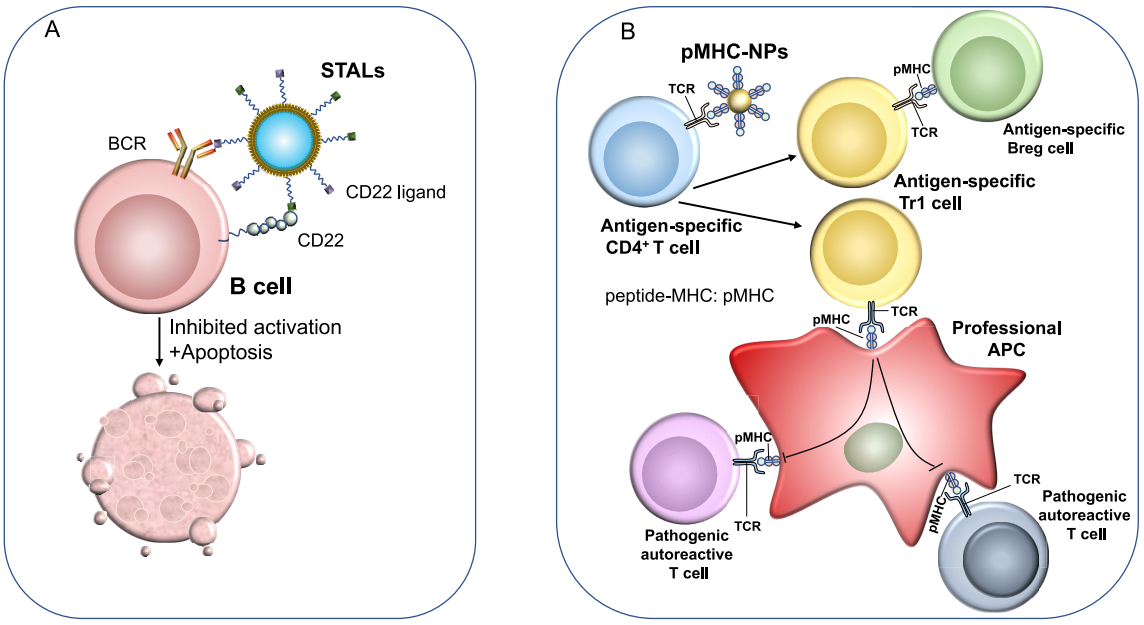

Figure 3. Particles reprogram the B cell and T cell directly to prime immune tolerance. (A) Schematic working mode of STALs, developed by Paulson, could induce B cell tolerance by activating apoptotic signaling pathways. Adapted with permission from ref 135. Copyright 2021 American Chemical Society. (B) NPs developed by Santamaria, which were coated with disease-relevant pMHC, trigger the differentiation of cognate autoantigen-experienced CD4$^+$ T cells into Type 1 regulatory T cells (Tr1) and lead to suppression of antigen-presenting and proinflammatory capacities and Breg cell formation. Adapted with permission from ref 136. Copyright 2019 Springer Nature.

harnessing this natural tolerogenic process.[102,103] Several groups showed the efficacy of antigen-coupled splenocyte-induced tolerance in autoimmune diseases and transplant rejection.[104−106] By conjugating antigens by ethylene carbodiimide (ECDI) fixation, this approach could effectively induce T cell anergy and peripheral tolerance and its safety and feasibility was determined in phase I clinical trials in MS patients.[107,108] However, the difficulty in GMP manufacturing, reproducibility in tolerance induction, and cost of splenocyte *ex vivo* collection and antigen−cell coupling have presented a big obstacle for further development.[52] Macrophages, located in the marginal zone of spleens, are found to be the main cell type responsible for recognizing apoptotic cells and tolerance induction.[100] This type of macrophage expresses high levels of the scavenger receptor type A protein, e.g., macrophage receptor with collagenous structure (MARCO).[109] By exploiting this mechanism while conquering the shortcomings of the aforementioned cell therapy, Miller and Shea et al. explored covalent coupling of antigens to polystyrene or polymeric NPs with size of ∼500 nm and proved the colocalization with macrophages expressing MARCO in the marginal zones of the spleen after iv injection.[85] Their NPs, delivering single or multiple antigenic peptides, have successfully primed antigen-specific tolerance in preclinical models of MS, T1D, allergic airway inflammation, and bone marrow transplantation.[110−113] The two characteristics of their NPs that matter the most in tolerogenic efficacy, by affecting the colocalization with MARCO, include NP size (∼500 nm) and negative surface zeta potential (∼50 mV).[85] Recently, Miller et al. developed TAK-101, a ∼500 nm negatively charged PLGA NP encapsulating gliadin (a component of gluten, which triggers immune-mediated disorder), and conducted phase 1 and 2 studies by iv administration for celiac disease.[114] Instead of coupling antigen onto the NP surface, they choose to encapsulate gliadin inside the NPs. NPs taken up by MARCO macrophages facilitate processing and presentation of the delivered antigen to gliadin-specific T cells. Then antigen-specific tolerance is induced mainly by activation of Foxp3$^+$ Tregs and IL-10-producing Tr1, and subsequent enhancement of the level of PD-L1 and secretion of TGF-β and IL-10. TAK-101 suppressed small intestinal mucosal inflammation injury, without arousing systemic immune suppression.[114] This clinical trial demonstrated the translatability of NP-based tolerogenic vaccines in autoimmune diseases.

The liver is well-known as the natural tolerogenic organ and plays a pivotal role in immune tolerance.[115] The liver not only inhibits unwanted immune responses against food antigens but also allows for tumor persistence and metastases.[116] Moreover, the liver tolerates hepatotropic pathogen chronic infection, e.g., hepatitis B/C virus, eventually resulting in systemic immune tolerance against viral antigens.[117] The liver also enjoys immune privilege during organ transplantation and requires less use of immunosuppressive drugs than heart or kidney transplantation.[118] The incidence of transplantation of a heart or a kidney accompanied by a liver is much higher than transplanting an organ alone since it is less prone to undergo immunological rejection.[119,120] Researchers studied the liver's immunosuppressive function and attributed it to its unique APC systems.[76] The liver has a large number of APCs featuring tolerogenic effects such as LSECs, KCs, liver resident DCs, and hepatocytes (Figure 2C). These APCs phagocytose foreign materials, degradation products, and toxins from sinusoidal blood and process them to prime tolerance instead of rousing inflammatory immune responses.[76] Researchers tried to explore the liver's ability and presented myelin basic protein (MBP) to the liver to control EAE to some extent in mice.[121] Among all the liver APCs, studies have demonstrated that LSECs are very prominent in (1) expressing low-to-undetected levels of T-cell co-stimulatory molecules, (2) constitutively

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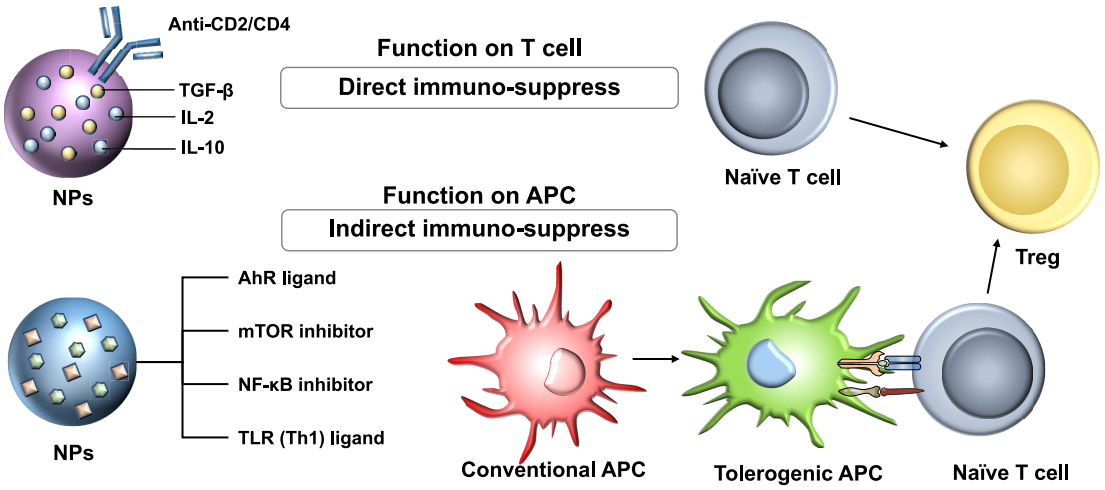

Figure 4. Particles co-deliver both antigens and immunoregulators to tune the immunomodulatory signals to promote immune tolerance. The upper panel depicts the co-delivery of antigens and anti-inflammatory cytokines to build a tolerogenic environment, which directly functions on the T cells and induces immunotolerance. The lower panel shows that pharmaceutical agents activate the tolerogenic signal pathways of APCs to indirectly induce immunoregulation.

secreting TGF-β, and (3) expressing co-inhibitory receptor (e.g., PD-L1) and inhibiting effector T cells.[75,76,122,123] Herkel et al. compared liver APCs and proved that LSECs were the most efficient in inducing Tregs and inhibiting effector T cells *in vitro*.[124] Moreover, they demonstrated that LSEC-induced neuro-antigen-specific Tregs are efficient suppressors in the EAE model *in vivo*, and this striking ability of LSECs was closely associated with TGF-β tethering to their membranes.[124] NP-based strategies to deliver antigens to LSECs proved to be more versatile and robust than conventional antigen delivery alone and cell-based therapies. NPs with size from ~200 to 500 nm are more likely to accumulate in the liver *in vivo*.[125] KCs phagocytose larger particles, while LSECs endocytose smaller particles and soluble macromolecules.[126,127] Herkel et al. attached antigen to polymer-coated superparamagnetic iron oxide NPs and facilitated rapid delivery of antigen to LSECs *in vivo*.[128] Such NPs provided potent alleviation for established diseases in the murine EAE model.[128] Exploiting differential scavenger receptor expression preference of LSECs, previous work in our group developed more selective LSEC-targeting NPs which facilitate delivery of allergens to LSECs *in vivo*.[77,129] The LSEC-targeting NPs could suppress allergic airway inflammation and induce TGF-β production with the occurrence of Foxp3$^+$ Tregs in the lung.[77,129] Therefore, targeted antigen delivery involving natural tolerogenic processes and inducing antigen-specific tolerance by NP-based vaccines represent a potent approach for autoimmune diseases and allergies.

2.3. Reprogramming B Cells and T Cells Directly to Prime Tolerance. Much attention has been paid to antigen-specific immune tolerance in the B cell arms of humoral immune responses. Paulson et al. have invented a direct strategy using STALs (Siglec tolerizing antigenic liposomes) to co-present allergens/autoantigens and CD22 glycan ligands (B cell inhibitory receptor Siglec) with high affinity on their fabricated liposome platform (Figure 3A). STALs target B cells and recruit CD22 to facilitate B cell receptor (BCR) antigen presentation.[130] This process results in B cell tolerance induction by activating apoptotic signaling pathways to kill antigen-reactive B cells and driving the subsequent depletion.[130,131] They have already demonstrated the successful application of STALs in animal models of disease involving the production of inhibitory antibodies to factor VIII in a hemophilia model mice and peanut-specific IgE in a peanut anaphylaxis model during the past years.[132−134] Recently they reported that the joint use of STALs and Selecta's rapamycin-containing NPs induced tolerance to long-term antigen challenges (5-weekly doses during a 150-day study period) in K/BxN mice, which develop spontaneous autoimmune arthritis to the self-antigen glucose-6-phosphate-isomerase (GPI), delaying disease onset and even reversing the disease.[135] Their study suggested the potential of targeting B cells to treat early stage autoimmune diseases.

The concept of nanoparticle-based "artificial/synthetic APCs" has also inspired the Santamaria group to construct particles that could directly function on T cells (Figure 3B). They focused on antigen-specific Treg expansion *in vivo* as the main point in the therapeutic intervention for autoimmunity. T cells would become anergic and differentiate into Tregs without the co-stimulatory signal 2.[136,137] Santamaria et al. constructed iron oxide NPs coated with T1D-relevant peptide−major histocompatibility class I complexes (pMHC-NPs) and treated nonobese diabetic (NOD) mice with pMHC-NPs.[138] The NPs functioned as synthetic APCs to present antigens without co-stimulatory molecules and lead to autoreactive CD8$^+$ T cells anergy along with antigen-specific Treg expansion. The results also showed that their treatment prevented the onset of disease in prediabetic mice and restored normoglycemia in diabetic mice.[138] It is worth noting that pMHC-NP therapy suppressed autoantigen presentation in an IFN-γ, indoleamine 2,3-dioxygenase-, and perforin-dependent manner.[138] Santamaria also applied their strategy to fabricate NPs decorated with peptide−major histocompatibility class II complexes.[139] They proved the efficacy of these fabricated pMHC-NPs in animal models of T1D, EAE, and arthritis.[139] This time they showed that pMHC-NPs activated and expanded antigen-specific Tr1. Moreover, the NP-expanded Tr1, in turn, suppressed autoantigen-loaded APCs and then primed the differentiation of cognate B cells into disease-suppressing regulatory B cells.[139] Their pMHC-NPs as tolerogenic vaccines represented a precise and personalized approach for a broad spectrum of autoimmune conditions.

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2.4. Tuning the Immunomodulatory Signals by Co-delivery of Immunoregulatory Agents. Three immunoregulatory signals are needed for tolerance priming and tolerogenic cell activation by NP-based tolerogenic vaccines. Inspired by the successful application of *ex vivo* induced tolerogenic DCs by particular pharmaceutical agents as prophylactic and therapeutic cell therapies,[140,141] this strategy could also be exploited by nanoparticle-enabled vaccines to ensure that APCs (1) are always embraced in the face of an anti-inflammatory environment, (2) differentiate or "lock-in" a tolerogenic phenotype,[142] and (3) fulfill the subsequent tolerogenic effector function. Since NPs have emerged as delivery systems for drugs (e.g., proteins, nucleic acids, small molecules, *etc.*) at the beginning and have been proven to be advantageous, they could be used as "off-the-shelf" vehicles incorporating both antigens/allergens and tolerogenic immunoregulators or pharmaceutical agents. The co-delivery of not only antigens but also immunoregulators guarantees NP-based vaccine specificity and also mitigates the risk of inflammatory responses and directs the antigen-specific immune cells toward tolerogenic function (Figure 4).

Leveraging this strategy, researchers directly co-administered anti-inflammatory cytokines and their mediators to shape a tolerogenic environment. Systemic lupus erythematosus (SLE), a common type of lupus, is an autoimmune disease in which Tregs have impaired function and no longer check self-reactive T/B cells.[143,144] Low dose therapies of selective cytokine modulators, for instance, IL-2 and TGF-β, have been used to amend Treg function and improve clinical symptoms in SLE patients.[145,146] La Cava et al. reported that PLGA NPs loaded with recombinant (r) IL-2 and TGF-β and conjugated with anti-CD4 antibody (for the targeting of $CD4^+$ T cells) could stably expand $CD4^+CD25^+Foxp3^+$ Tregs *ex vivo*.[147] They then co-conjugated NPs with anti-CD2 antibodies and extended the targeted delivery of rIL-2 and rTGF-β for both $CD4^+$ and $CD8^+$ Tregs *in vivo* and also suppressed lupus manifestations in a mouse model of SLE, resulting in a reduction of specific autoantibodies and immune-complex glomerulonephritis.[147] Cappellano et al. used PLGA NPs with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ autoantigen and rIL-10 encapsulated inside for both subcutaneous prophylactic and therapeutic vaccination in a mouse EAE model.[148] The particles co-loaded with rIL-10 and autoantigen induced decreased histopathologic lesions in the central nervous system tissue with reduced production of IL-17 and IFN-γ.[148] For this strategy, the scale-up of GMP production of multiple cytokines might be costly and become an obstacle to further clinical advancement.

Besides the direct administration of nanoparticle-based cytokine delivery to APCs shaping an anti-inflammatory setting, the delivery of pharmaceutical agents for the activation of the tolerogenic signal pathway in APCs also contributes to immunoregulation. Quintana et al. found that the ligand-activated transcription factor aryl hydrocarbon receptor (AhR) activated by the nontoxic mucosal ligand 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), could promote the differentiation and expansion of tolerogenic DCs and $Foxp3^+$ Tregs in mice and humans.[149,150] The activation of the AhR signaling pathway by ITE also showed the suppression of EAE in mice.[150] Attracted by the unique features of NPs, they used gold NPs with a diameter of ~60 nm coated with ITE and MOG_{35-55} on the NP surface.[151] The ITE-containing NPs activated an AhR-responsive promoter cell line *in vitro*, while NPs without ITE could not activate the AhR-responsive promoter. Moreover, the ITE-containing NPs improved suppression of the development of EAE with alleviated EAE symptom scores and reduced encephalitogenic Th1 and Th17 T-cell responses.[151] Recently their group explored nanoliposomes (NLPs), an alternative type of NP, loaded with ITE by insertion within the lipid bilayer, and MOG_{35-55} epitope within the hydrophilic liposome core, were ~100 nm in average size and negatively charged.[152] *In vivo* studies demonstrated that NLPs ameliorate acute EAE and the induced tolerance is long-lasting when administered one month prior to disease induction. They found the reduced production of proinflammatory cytokines (IL-17, IFN-γ, and TNF-α) with increased IL-10 level, and detected MOG_{35-55}-specific $Foxp3^+$ and IL-10^+ $CD4^+$ T cells in the central nervous system.[152] The Quintana group has performed many studies contributing to AhR signaling which modulates APC function and differentiation of T cell subsets.[152−154] The NP-based platforms were brought forward by them as candidate antigen-specific therapeutic approaches for the management of autoimmune disorders.

Rapamycin, an immunophilin ligand with potent immunosuppressive properties, has been explored as a nanoparticle-based therapy to induce antigen-specific tolerance in autoimmune diseases and allergy.[155,156] Rapamycin inhibits the function of mammalian target of rapamycin (mTOR), a serine/threonine kinase in the signaling pathways regulating cell proliferation and protein translation.[157,158] Thomson et al. showed that rapamycin induced alloantigen-specific T cell unresponsiveness *in vivo* by inducing a tolerogenic DC phenotype and Treg differentiation which was even resistant to triggering of the inflammatory toll-like receptor (TLR) signal.[159,160] The immunosuppressive activity and efficacy of rapamycin make it a potentially applicable strategy in nanoparticle-based tolerogenic vaccines. However, rapamycin therapy often requires long-term systemic injection, and thus there is an unmet need for more versatile and clinically applicable methods to decrease dosages and side effects by avoiding systemic broad immunosuppression.[161,162] Kishimoto et al. developed PLGA tolerogenic NPs co-encapsulating protein/peptide antigens and rapamycin and demonstrated that either sc or iv administration of their NPs suppressed both antigen-specific T cell and B cell responses, meanwhile promoting the induction of antigen-specific Tregs and Bregs.[156] The long-lasting effect of NP treatment was also detected up to 234 days, and the NP-treated animals showed inhibition of anti-OVA responses ~30-fold lower than the untreated groups during the experiment period.[156] The fate of the NPs was determined after injection *in vivo*, and the NPs mainly accumulated in the lymphoid organs including the spleen and draining LNs. The prophylactic treatment with NPs in the EAE model inhibited the onset of disease, and therapeutic treatment by a single shot of NPs achieved complete inhibition of disease relapse.[156] Besides, they applied the rapamycin-containing NPs in an allergic airway model, and NPs were effective in preventing IgE-mediated anaphylaxis. Also, they expanded the use of NPs to prevent antidrug antibodies and co-injection of NPs and free biologic drugs succeeded in models of hemophilia A, rheumatoid arthritis, Pomp disease, mesothelioma, adeno-associated virus gene therapy vectors, and pegylated uricase (pegadricase).[163−167] The NPs with rapamycin (no antigens) and pegadricase completed phase 2 clinical trials (NCT02959918) for

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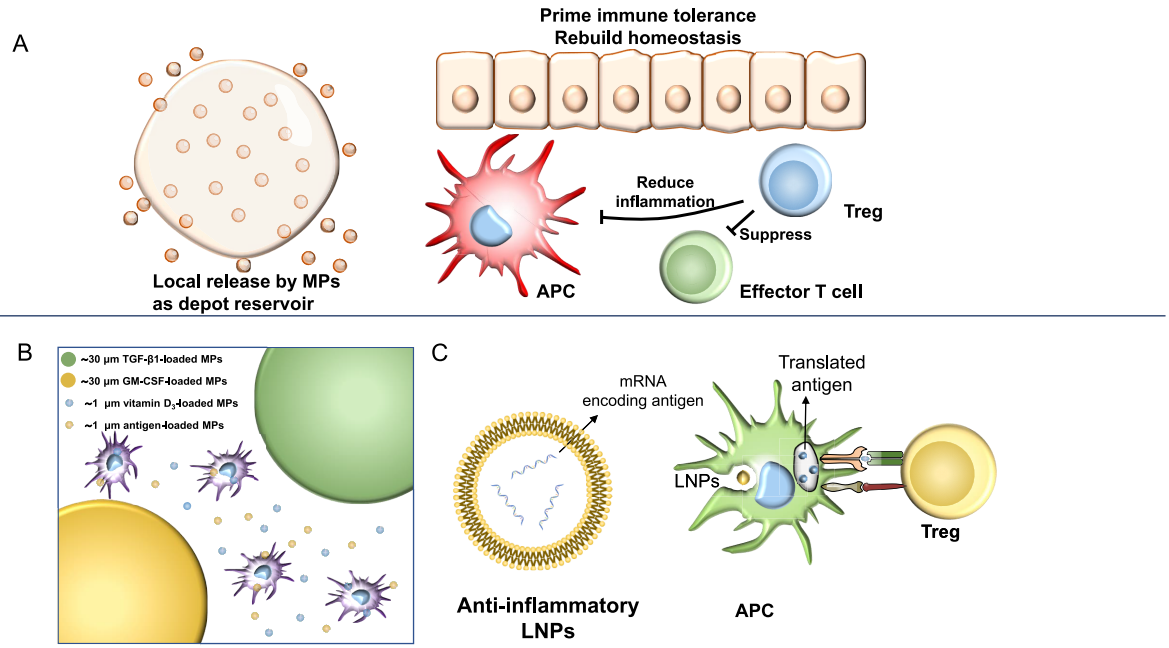

Figure 5. MPs, as antigen depot reservoirs, remain at the injection site and reprogram the immune cells *in situ*. The induced Tregs could reduce APC inflammation and suppress the effector T cells, therefore priming immune tolerance and rebuilding homeostasis (A). Dual-sized MP consisting of two types of MPs with different sizes, could recruit and condition APCs while providing intracellular antigen delivery to APCs to promote tolerance (B). Adapted with permission from ref 62. Copyright 2019 American Chemical Society. Anti-inflammatory mRNA vaccine, composed of LNPs containing mRNAs encoding specific antigens, could trigger the proliferation of Tregs and mediate antigen-specific suppression (C).

multidose safety/pharmacodynamic study with patients having symptomatic gout and elevated blood uric acid in early 2022[168] and are currently advancing to the phase 3 clinical trials (NCT04513366) for determination of safety and efficacy of two different dose levels in patients with refractory gout.

NF-κB, a transcription factor found in all nucleated cells, is important in regulating inflammatory immune responses, especially by cytokine production.[169] APCs, especially DCs, in the condition of immune disorders, were shown to have constitutive NF-κB activation posing an obstacle to tolerance induction.[170,171] The NF-κB subunit RelB-deficient splenic DCs have been proven to be refractory to TLR-ligand activation, and by adoptive transferring RelB-deficient DCs, murine autoimmune arthritis has been suppressed.[172−174] Rheumavax, the autologous DCs modified with an NF-κB inhibitor BAY11-7082 and exposed to four citrullinated rheumatoid arthritis peptide antigens, was in human phase 1 clinical trial of single intradermal injection.[175] A reduced inflammatory T cell IL-6 response and an increased ratio of Tregs were detected.[175,176] With this clinical proof of concept and the advantage of particle-based delivery for precise interaction with interested APCs and avoidance of the risk of activating effector T cells or prohibition of unrelated cell function by delivery of free antigens or NF-κB inhibitor, Thomas et al. coencapsulated antigens with multiple NF-κB inhibitors, including curcumin, quercetin, and 1α,25-dihydroxy vitamin D3 (calcitriol), into liposomes against autoimmune diseases.[177−179] These liposomes induced antigen-specific Treg expansion, inhibited the effector function of bystander $CD8^+$ T cells, and suppressed the development of rheumatoid arthritis and diabetes.[177,179]

It has been shown that TLR-mediated inflammation strongly exacerbates autoimmune diseases, such as in the EAE model.[180−182] CpG is potent as an adjuvant to induce antibody production and strong Th1 responses by binding TLR-9.[183] By employing this attribute of redirecting a Th2 toward a Th1 response, CpG has been used in the allergic asthma model and successfully prevented allergic airway inflammation by inhibiting Th2 cytokine induction, airway eosinophilia, and bronchial hyperreactivity.[184,185] On the other hand, for Th1-mediated autoimmune diseases, for instance, the EAE model, Steinman et al. demonstrated that a simple substitution of guanine for the cytosine in the CpG oligodeoxynucleotide (ODN) motif could skew both the autoaggressive T and B cell responses into a protective Th2 cytokine pattern and an IgG1 isotype, respectively.[186,187] Inspired by the aforementioned success of using CpG in tolerogenic therapies, Jewell et al. coassembled the autoantigen peptide myelin and CpG into particles with polyelectrolyte multilayers, and these NPs blunted myelin-triggered inflammatory responses, expanded Tregs, and inhibited disease in a MS model.[188] Their nanoparticle-based platform, involving no synthetic polymers as components of the carriers, could be versatile with tunable cargo loading and co-delivery features. And this platform could be a potential nanotherapy to induce tolerance by altering TLR function.

3. MPs PLAY A NOTABLE ROLE AS TOLEROGENIC VACCINES TO PRIME ANTIGEN-SPECIFIC TOLERANCE

MPs, having larger size ranges and payload capacities, could be unable to directly enter the wanted organs (e.g., lymphatics or lymph nodes) and mostly remain at the injection site and function as immunomodulatory depots to create or maintain the local tolerogenic microenvironment. This could enable the differentiation and direct the function of immune cells in these

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tissues, and the subsequent immune tolerance generation in organs of interest or even systemic tolerogenic function.

3.1. Forming Immunomodulatory Depots to Modulate the Local Microenvironment. MPs possess the benefits of a depot to release antigenic and tolerogenic signals facilitating a tolerogenic microenvironment (Figure 5A). Jewell et al. applied a direct intranodal injection (i.e., LNs) strategy to deliver MPs co-encapsulating MOG and rapamycin after induction of EAE.[189] Their single LN injection prevented the appearance of EAE and promoted increasing Treg infiltration since the MP depots provided local controlled release of both antigen and immunoregulatory signals in the injected node upon the particles' slow degradation.[189] In their experiments, NPs caused a lower effect compared to MPs because their smaller size allowed more efficient drainage from the lymph node without the establishment of a tolerogenic microenvironment.[189] Moreover, the synergistic effect from MP-enabled co-delivery of MOG and rapamycin enhanced the inhibition of EAE symptoms. They also proved the antigen-specificity of their depot-mediated tolerance by administration of MPs loaded with OVA and rapamycin which showed no effect on EAE symptoms.[189,190] To focus on the deficiency of Treg expansion *in vivo* during autoimmune diseases, Green et al. loaded rapamycin into particles, then conjugated the with tetrameric MHC class II/myelin peptide complexes and IL-2 fusion protein, to generate tolerogenic MPs.[191] Moreover, their MPs could reeducate immune cells to regard autoantigen as "harmless" instead of "foreign" for peripheral tolerance induction. The results showed the validated efficacy in clinically symptomatic EAE with long-term progression reversal and full recovery of 38% of the mice.[191] They envisioned that their platform with *in situ* antigen-specific Treg-activation and expansion ability could have great potential as immunotherapy for many autoimmune conditions.

3.2. Mimicking Artificial Immune Organs to Reprogram the Immune Cells *In Situ*. MPs can be also engineered as an "artificial immune organ", remaining at the injection site and reprogramming the immune cells *in situ* (Figure 5B). Keselowsky et al. designed and built their combination dual-sized controlled release MP system containing two sizes of MPs: (1) non-phagocytosable ~30 μm MPs loaded with pharmaceutical agents to modulate a localized tolerogenic microenvironment, for instance, granulocyte−macrophage colony-stimulating factor (GM-CSF) for DC chemokine and pro-tolerogenic factor cytokine TGF-β1; (2) phagocytosable ~1 μm MPs loaded with disease-relevant antigens or drugs as intracellular delivered targets (e.g., vitamin D3).[62,192,193] Their systems prevented the onset of T1D in NOD mice and even reversed T1D in the nonobese diabetic mouse model. In the EAE mouse model, a reduction of inflammatory pathogenic $CD4^+$ T cells was detected, suggesting a potential potent multifactor combinatorial approach.[62,192] Giannoukakis et al. simplified a similar concept into a single MP formulation composed of all-trans retinoic acid (RA) and TGF-β with conjugated insulin peptide B9−23 on the particles' surface.[194] RA and TGF-β based immunoregulatory concepts have been demonstrated for their feasibility to suppress inflammation and enhance tolerance.[195,196] The MPs could prevent the progression toward overt diabetic clinical hyperglycemia when injected into early midstage NOD mice. They also found the possible engagement of regulatory B cells without the elevated induction of Treg frequency.[194] Compared to the multiple particle population developed by Keselowsky, the "three-in-one" single MPs might require complex fabrication and achieve demanding characterizable physicochemical properties. The comparison between both MP design concepts for various autoimmune diseases or under different stages should be considered to optimize the outcomes of regulatory induction.

3.3. Promoting Homeostasis Both Locally and Systemically. MPs not only can fulfill their function as depots by releasing their immunoregulatory payloads and antigens but also can play a role in immune homeostasis. Chen et al. developed MPs that rapidly released monocyte chemotactic protein-1 to recruit activated autoreactive T cells and induce apoptosis of these cells by the Fas ligand (FasL) coupled on MPs' surface.[197] They found that the digestion of apoptotic T cells by macrophages could induce the subsequent TGF-β production and Treg differentiation. The MPs encapsulating autoantigens were tested in the murine models of EAE and nonobese diabetes and caused disease reduction successfully by establishing an immune-tolerant environment.[197] Since FasL-expressing killer APCs could eliminate antigen-specific T cells through apoptosis induction, artificial FasL-expressing killer APCs have been constructed by conjugating MHC complex with the anti-Fas mAb onto magnetic beads. Zhang et al. coupled H-2Kb/peptide monomers and anti-Fas mAb to latex beads and injected them into BALB/c mice with prior grafted skin squares from C57BL/6 mice to test tolerance induction.[198] The alloskin graft survived for 6 days and a decrease of antigen-alloreactive T cells was detected.[198] Shen et al. fabricated PEI-coated PLGA MPs co-coupled with H-2Kb−Ig dimer and anti-Fas mAb, and their MPs prolonged the alloskin graft survival up to 43 days and depleted most of the H-2Kb-alloreactive $CD8^+$ T cells in the alloskin graft, peripheral blood, and spleen without impacting native T cell repertoire and overall immune function.[199]

4. PARTICLE-BASED NUCLEIC ACID TOLEROGENIC VACCINES

Nucleic acid vaccines consisting of DNA or mRNA molecules, when injected and internalized by target immune cells, can manipulate the cell machinery to express the gene of interest, translate it into targeted protein products, and promote the whole process by co-coupling a highly active promoter with the coding sequence.[200−202] The DNA or mRNA molecules could encode both antigens and desired immunomodulators. The self-amplifying mRNA (SAM) mediates mRNA autoamplification and not only translates into wanted antigens but also further enhances antigen expression.[202] In addition to antigens, co-delivered immunomodulatory protein/peptide molecules could also be encoded and expressed, which include TGF-β, IL-10, etc. Karin et al. showed that the combinatorial injection of plasmid DNA vaccines encoding IL-10 together with a plasmid encoding MBP in EAE model rapidly induced significantly improved suppression of ongoing disease compared to the plasmid DNA encoding MBP alone.[203] Disease-specific tolerance included an increase in antigen-specific IL-10- producing T cells as well as an increase in cell apoptosis in the central nervous system.[203] The simple and versatile manufacturing process of nucleic acid could facilitate removing intrinsic immunostimulatory sequences and excluding the unwanted responses, for instance, the removal of the IgE-binding peptide domain to abrogate IgE binding and subsequent activation and other prevention strategies to

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eliminate TLR-3/7/8 stimulation to replace the related motif domain with 1-methyl pseudouridine. The recent application of mRNA vaccines to combat the COVID-19 pandemic has prevented and alleviated the severity and morbidity of SARS-CoV-2 infection.[204] Sahin, who led the BioNTech company to develop the COVID-19 mRNA vaccine, has now harnessed this technology to develop a tolerogenic vaccine.[205] Working with mouse models of MS, he and colleagues showed that LNPs with a modified mRNA could lead to the suppression of autoimmune responses through the occurrence of antigen-specific Tregs (Figure 5C). Since in humans several autoantigens could drive MS instead of just one type of autoantigen, they designed mRNA encoding a mixture of four autoantigens and tested it in a particular EAE model primed with five autoantigens.[205] The results showed the same protection efficiency achieved by their mRNA vaccine.[205] They are currently carrying on further research to assess the clinical potential of their strategy, hoping to transform their approach for other autoimmune diseases.

Very recent studies showed a LNP platform to target and deliver mRNA-encoded peanut allergen epitopes to LSECs to induce immune tolerance.[206] The nucleotide sequences encoding for nonallergenic MHC-II binding T-cell epitopes were included. The mRNA strand was designed by inserting single tandem or combined epitope sequences downstream of a MHC-II routing sequence.[206] Using codon-optimized mRNA, the LNPs were synthesized by microfluidics with a mannose ligand decorated on the surface for LSEC targeting. They confirmed the LNPs' biodistribution to the liver, and the efficacy of LNP treatment for food allergen anaphylaxis was also demonstrated with significant alleviation of physical anaphylaxis manifestations, and suppression of IgE production and Th2-mediated cytokine, mast cell release, along with elevated TGF-β and IL-10 levels in the peritoneum.[206] Those findings exhibit a potential application of the mRNA/LNP platform with the ability to prevent anaphylaxis caused by food allergens, with the potential to be widely applicable to other allergies.

5. DEVELOPMENT OF ANTIGEN IDENTIFICATION FOR PARTICLE-BASED TOLEROGENIC VACCINES

The antigen used in tolerogenic vaccines has been through blooming development during recent years with the aid of recombinant protein and DNA technology, including characterization of the active contents and related biological activities of the complex antigens, and prediction and molecular dynamic stimulation of the main antigens. In earlier years, for most important antigens, the defined recombinant antigen molecules have been expressed using isolated cDNAs. Recombinant antigen microarrays assist in defining the reactivity profiles of antigens and provide possibilities for tailoring individual needs.[207,208] At present, several treatment strategies to obtain engineered optimal antigens have been applied and pursued in preclinical and clinical studies involving recombinant antigen oligomers or engineered antigen derivatives and recombinant hybrid antigen proteins.

Recombinant antigen oligomers, which have been expressed by several copies of the cDNA coding one particular antigen or the cDNA coding several antigens or antigen epitopes in one expression vector, are emerging usually as genetically engineered protein dimers and trimers.[209−211] When injected for therapeutics, a recombinant trimer of Bet v1, a polypeptide of the main birch-pollen allergen, was more immunogenic than its fragments.[209,212] Recombinant hybrid antigen proteins, consisting of antigen complex in one single molecule, have been proven to show increased immunogenicity compared with the separate molecules.[213,214] For instance, a hybrid allergen protein containing three main allergens in one molecule is hypoallergenic and preventative against bee venom allergic sensitization and challenge in a murine model.[213]

Among all the aforementioned strategies for engineered recombinant antigens, most researchers pointed out their epitopes generated from T cells with reduced IgE reactivity.[215−217] In contrast to intact antigens, this T-cell-epitope-containing peptide approach immunomodulates T cells with less ability to cross-link IgE to activate effector responses. Phage-display technology, enabling gene encoding of a particular protein and display on the bacteriophage surface, has been applied to link the genotype and phenotype of antigens physically.[218] Therefore, the peptides of antigens that are recognized by antigen-specific IgE could be isolated by phage-display technology, and the subsequent molecular modeling could facilitate further selection of antigens excluding IgE-binding epitopes.[219,220] The epitope mimics (known as mimotopes) could also partially inhibit the high-affinity interaction between antigen epitopes and the related IgE.[218,219] The more potent clinical efficacy of this approach has been verified to be associated with moderate adverse events after allergen challenge, as a result of avoiding IgE-cross-linking peptide residual tertiary structure, such as reducing allergic skin reactions during the middle/late phase and reducing airway hypersensitivity responses.[221−223] T-cell epitopes, with growing interest, represent antigenic peptides interacting with MHCII on APCs and inducing ensuing potential differentiation to Tregs. The research group of Sampson and O'Hehir has identified the dominant human T-cell epitopes for three main peanut antigens (Ara h1, Ara h2, and Ara h6), and developed epicutaneous therapies of T-cell epitopes for treating peanut anaphylaxis.[224−227] For NP-based tolerogenic vaccines, our group has deployed the Immune Epitope Database and Analysis Resource (http://tools.iedb.org/mhcii/) to search for candidate T-cell epitopes, combined with additional peptide analysis based on particle encapsulation efficiency.[228] The epitope physicochemical properties can be assessed by applying an isoelectric point (pI) calculator (https://web.expasy.org/protparam/), and a tool of "grand average hydropathicity values" (GRAVY) to predict the hydrophilicity/hydrophobicity. Screening of the selected peptides would then be tested for the ability to prime Foxp3$^+$ Treg expansion *ex vivo*.[228] The selection and use of Ara h2 T-cell epitopes in a particle-based tolerogenic vaccine have generated protection to prevent peanut anaphylaxis.[228] In the near future, an AI-assisted approach will be established to facilitate the systemic and optimal antigenic selection of T-cell epitopes, particularly for particle-based tolerogenic vaccines.

The mRNA construct design has also been studied by our group based on the selected epitopes. To allow for insertion into the endoplasmic reticulum membrane of MHCII compartment, the antigen epitope sequence was designed for inserting the tandem-repeat unit downstream of an invariant li chain sequence.[206,229] Moreover, to avoid unwanted immune stimulatory responses, they replaced with N1-methyl-pseudouridine in the nucleotide backbone. The particles have been proven to target and deliver the as-constructed mRNA to LSECs to treat peanut-induced anaphylaxis in a murine

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Table 2. Clinical Trials on Particle-Based Tolerogenic Vaccines for Antigen-Specific Tolerance Induction

clinical application	phase	particle properties	administration route and	outcome refs
celiac disease	phase 2	PLG nanoparticles encapsulating gliadin protein	intravenous infusion	https://www.clinicaltrials.gov/study/NCT03486990
gout chronic hyperuricemia	phase 3	pegsiticase (pegylated uricase) and PLGA particles consisting of rapamycin	intravenous infusion	https://www.clinicaltrials.gov/study/NCT04596540
type 1 diabetes	phase 1	gold nanoparticles with a proinsulin derived peptide	administered intradermally by microneedles	https://clinicaltrials.gov/ct2/show/NCT02837094
rheumatoid arthritis	phase 1	liposome encapsulating collagen II 259−273 (CII) peptide co-delivered with NF-κB inhibitor, 1,25-dihydroxycholecalciferol (calcitriol)	subcutaneous administration	2019 ACR/ARP Annual Meeting Archives/Abstracts 5T092: RA Treatments IV: Novel Therapy & Predicting Response (Abstracts 2768−2773)
multiple sclerosis	double-blind	capsules of bovine myelin or control protein	oral administration	*Science* **1993**, *259* (5099), 1321−4. doi: 10.1126/science.7680493

model.[206,230] For the future use of mRNA constructs in particle-based tolerogenic vaccines, careful consideration of design features would be necessarily required to achieve the best potency as well as rule out possible unfavorable effects.

6. CONCLUSION AND PERSPECTIVES

Tolerogenic vaccines have significant potential as immunotherapies with various benefits for autoimmune diseases, allergy, and transplantation rejection. The targeting ability to organs/cells of interest and inclusion into natural tolerogenic processes, directly impacting B and T cells, co-delivery of antigens and pharmaceutical molecules to boost tolerance, and larger MPs as depots to restore immuno-homeostasis have collectively enhanced the efficacy of tolerogenic vaccines. Currently, several particle-related vaccines are reaching or completing clinical studies (Table 2).

Most current tolerogenic vaccines have been rationally constructed by leveraging the known properties of NPs/MPs along with antigens/allergens to realize predefined functions. The accelerated discovery of biotechnology and the advancement of materials and chemistry speed up the development of tolerogenic vaccines. However, numerous antigens/allergens require customized particles for delivery, and obtaining optimal particles as delivery vehicles can be laborious and tedious. Extensive production and application of NPs are the hottest trends that boost nanomedicine and therapy toward rapid development. The rate and dynamics of both academic research work and clinical experiments for developing particle-based tolerogenic vaccines urgently call for consideration of predictive nanobiology working paradigms. NPs' intrinsic properties including their material composition, size/size distribution, shape, and surface coatings/functions could dictate NPs' fate and their nanoimmunological communications. NPs, after entering the body, could be located in different organs and emerge to interact with immune systems, particularly APCs. The interaction of NPs and APCs determines the subsequent immune responses. Based on our previous research experience on engineered nanomaterials (ENMs) and their related biological responses, the prediction between NPs and APCs including cellular and molecular level screening could be of great importance.[231,232] Typical tolerogenic APCs and T-cell coculture evaluation systems could be used and optimized based on the choice of APCs and the tested particles and focused on immune suppression and anti-inflammatory effects which include particle recognition and internalization, APC activation and maturation, antigen processing and presentation, co-inhibitory surface receptor expression, production of anti-inflammatory cytokines and chemokines influencing T-cell polarization, immune modulation and editing effects, etc.

The ideal prediction will determine the particle physicochemical properties and immunological activities established on the high-content screening or high-throughput sequencing which could monitor the whole stages of immune response.[233] The high-throughput evaluation assays should be used as a predictive assays to determine the *in vivo* performance of the synthesized particles and decrease the subsequent testing in animal studies. Taking gene expression analysis as one typical example, analyzing gene expression levels for profiling of the signal pathways associated with autoimmune diseases receiving NP-based tolerogenic vaccines, allows for progression analysis of NP-directed therapeutic vaccines at different disease stages, identification of particle working mode or mechanism to modulate the disease, and prediction of particle treatment which would benefit other autoimmune diseases. Numerous gene expression analysis assays have been developed. qRT-PCR has been used to amplify 1−50 genes of interest, and FISH (Fluorescence *In Situ* Hybridization) has been used to hybridize one tissue sample using fluorescent-labeled short DNA probes to localize and detect the target sequences *in situ*.[234,235] With the assay reproducibility, specificity, sensitivity, throughput, and complexity improving rapidly, the following technologies are currently often in use: the NanoString system detects usually 50−500 genes using probe annealing pairs; the RNA-seq system sequences the mRNA molecules in the whole transcriptome; tissue microarrays determined the expressed gene directly from hundreds of tissue sample blocks.[234,236−238] Such assays are quite useful tools for us to facilitate the use of particles as tolerogenic vaccines in autoimmune diseases and other immune disorders. Besides, cutting-edge spatial multiomics technologies have been used to provide spatial information for cell-to-cell interactions along with the data obtained from multiparameter or multidimensional analysis (e.g., multiplex immuno-histochemistry/immuno-fluorescence).[239,240] Given the complicated properties of NPs and complexity of the immune system, those high-throughput approaches and evaluation methods are urgently required to establish quantitative structure−activity relationships for future tolerogenic vaccines.

Since entering the AI era, rapid progress in the development of intelligent analytic and computational technologies has provided many valuable insights for revealing mechanisms and effects of complex nano−bio activities and interactions. Furthermore, machine learning algorithms should also be adopted to predict particle-based tolerogenic vaccine synthetic outcomes. As mentioned above, under AI assistance, the design

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properties (e.g., size, shape, and surface modifications) and related parameter ranges for particle fabrication could be significantly narrowed down to realize the rapid procurement of optimal particle vaccines. The widely used machine learning models, such as random forest, artificial neural network, support vector machine, and meta-analytical workflow concepts, have been through exploration by researchers to understand the bio−nano interactions among multiple feature interactions.[241−244] Further refining the model interpretability and the automatic degree of interaction coefficient calculations will also allow machine-learning approaches toward big data to facilitate particle-based tolerogenic vaccines.

In recent years, various types of EVs secreted by cells have been reported as emerging techniques, especially for nanomedicine.[245] EVs with membranous structures consisting of lipid bilayers could be capable of enhancing cell-to-cell communication and modulating multiple physiological and pathological processes like tumor metastasis, microenvironment homeostasis, and immune regulation.[246,247] For autoimmune disease or allergy scenarios, Treg-derived EVs may represent a refined intercellular tolerance-inducing device. Its proposed mechanism includes microRNA gene silencing, interaction of surface proteins, and transmission of enzymes.[248,249] Treg EVs are unlikely to be modified even after exposure to an as-constructed inflammatory microenvironment,[249] which highlights the benefit of their use for intervention with T cell differentiation in autoimmunity treatment. Despite the ability to escape phagocytosis and achieve long-term circulation and current promising therapeutic results in animal studies, EV-based vaccines and delivery systems still have huge challenges in manufacturing and clinical translation.[250] The laborious process including synthesis, purification, modification, loading, and storage and the heterogeneity between batches, sources, and subpopulations would greatly hinder the quality control for the development of EVs.[250,251] Rising numbers of studies are using nanobiotechnology to remodel EVs to overcome these shortcomings.[252,253] The nanofabrication of these artificial EVs mainly includes (1) top-down strategies by manipulating cells with microfluidic devices, disrupting cells by sonication/nitrogen cavitation or under alkaline solution; (2) bottom-up strategies by generating EVs by supramolecular chemistry and synthetic biology; (3) biohybrid technology by fusing EVs with liposomes and LNPs to form hybrid EVs without affecting the intrinsic properties.[253] These would hold great promise for tolerogenic vaccines with the combined advantages of EVs and synthetic NPs.

The use of redirected T cells and chimeric antigen receptors (CARs), besides extraordinary success in FDA-approved cancer therapies, holds promise to provide robust cell therapies to induce antigen-specific tolerance. For the known autoantibody targets, the strategy of chimeric autoantibody receptors (CAARs) or B cell antibody receptors (BARs) on cytotoxic T cells, selectively ablate only B cells expressing antigen-specific receptors.[254,255] In the cases of unknown autoantibody targets, anti-B cell CAR T cells completely and sustainably deplete B cells, unlike mAbs. CAR Treg cells are an alternative approach to target Tregs for local induction of tolerance.[256,257] NP-based tolerogenic vaccines could also be used in combination with CAR-based therapies. *In situ* reprogramming, adapted to the combination of CAR and particles, emerging as a potentially disruptive technology in this field, targets T cells and expresses a CAR in the body, to achieve the goal of T cell reprogramming.[258,259] NPs loaded with CAR-encoded mRNA, along with precise targeting ability, could be internalized by T cells. The subsequent production of CAR T cells in the body would be realized by translation of the delivered mRNA, protein assembly, and transportation to the cell membrane.[258] The interesting collaboration holds the promise of cell therapy while obviating *ex vivo* manufacturing, thereby making a giant step forward to improve scalability, lower cost, and increase access. Moreover, particle-based tolerogenic vaccine *in situ* reprogramming using the CAR T cell strategy could be applied spatially. Briefly, anti-APC CAR T cells could ablate autoantigen-specific APCs, CAR Tregs could be targeted to the local inflammatory organs for direct suppression, and anti-B cell CAR T cells could be used to deplete autoantibody-producing cells.[254] The exciting potential is ahead of us and encourages us to make efforts to extend these advances.

Particle-based tolerogenic vaccines combined with other modalities, for instance, therapeutic mAbs, are anticipated to be future directions due to their complementary benefits. This is in agreement with the impact on the factors integrating the whole immune tolerogenic microenvironment. Moreover, by applying the aforementioned high-throughput approaches, multiomics, and machine learning to handle big data, it could be advantageous to identify proper biomarkers and establish an evaluation system for future personalized and precision particle-based tolerogenic vaccine development. The evaluation system could be beneficial, especially for non-invasively quantified or imaged autoimmune disease scenarios.

In conclusion, the power and potential of particle-based tolerogenic vaccines in modulation of the immune system to combat autoimmune diseases and allergy are among the most exciting advances in immunotherapy. Effort will be made toward effective, safe, widely available, and affordable tolerogenic vaccines as the ultimate goal. Thus, it would be quite possible to see major advances in particle-based tolerogenic vaccines or related immunomodulatory therapies in the near future.

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Notes

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VOCABULARY

Immune tolerance, a highly regulated process to prevent unwanted immune responses against self-antigens and non-harmful foreign antigens; Tolerogenic vaccines, antigen-specific tolerogenic immunotherapy by suppressing pathological immune responses to specific allergens/autoantigens; Autoimmune diseases, a diverse group of pathophysiological conditions wherein aberrant immune response are caused to damage the body's healthy tissues and normal constituents; Anaphylaxis, an acute, severe, potentially life-threatening systemic hypersensitive reaction triggered by specific allergens; Regulatory T cells, a distinct subset of T cells regulating and suppressing both innate and adaptive immune responses to promote immune tolerance and maintain immune homeostasis

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