Rational Vaccinology: Harnessing Nanoscale Chemical Design for Cancer Immunotherapy

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Cite This: ACS Cent. Sci. 2022, 8, 692–704

ABSTRACT: Cancer immunotherapy is a powerful treatment strategy that mobilizes the immune system to fight disease. Cancer vaccination is one form of cancer immunotherapy, where spatiotemporal control of the delivery of tumor-specific antigens, adjuvants, and/or cytokines has been key to successfully activating the immune system. Nanoscale materials that take advantage of chemistry to control the nanoscale structural arrangement, composition, and release of immunostimulatory components have shown significant promise in this regard. In this Outlook, we examine how the nanoscale structure, chemistry, and composition of immunostimulatory compounds can be modulated to maximize immune response and mitigate off-target effects, focusing on spherical nucleic acids as a model system. Furthermore, we emphasize how chemistry and materials science are driving the rational design and development of next-generation cancer vaccines. Finally, we identify gaps in the field that should be addressed moving forward and outline future directions to galvanize researchers from multiple disciplines to help realize the full potential of this form of cancer immunotherapy through chemistry and rational vaccinology.

1. INTRODUCTION

Cancer immunotherapy trains the immune system to seek and destroy cancer cells with high specificity, in stark contrast to traditional approaches that aim to “cut, poison, and burn” tumors. Indeed, conventional methods, including surgery, chemotherapy, and radiation, are fraught with drawbacks. Serious side effects often arise from insufficient tumor targeting, and in addition, the dynamic nature of tumor development often inhibits efficacy. For example, drug resistance and off-target effects limit the action of chemotherapeutics, and localized treatments (e.g., in situ lesion treatment, surgical resection, and radiation) cannot generally be used to control tumor metastasis. Thus, cancer immunotherapies represent an important paradigm shift in cancer treatment because these methods mobilize the immune system to seek and destroy existing cancer cells at both primary and distal sites and, in some cases, induce strong, protective immune responses to prevent future tumorigenesis.

The concept of deploying the patient’s own immune system to fight cancer dates back to at least the 19th century and has relied on the administration of “non-self” biomaterials to activate immune cells to attack tumors. In the 1890s, Dr. William Coley reported instances of cancer remission following intratumoral injections of deactivated bacterial mixtures, known as “Coley’s toxins.” Coley’s toxins consisted of a mixture of proteins, carbohydrates, nucleic acids, and lipids from different bacteria. These mixtures stimulated the innate immune system and were “designed” to give patients severe fevers and simultaneously shrink their tumors. Over the following century of research, it was determined that the tumors were not eliminated by the “toxins” but rather killed by immune cells that they had activated. Indeed, a fundamental principle of modern cancer immunotherapy involves activating antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages, and B cells, and training them to attack tumors through innate and adaptive immune responses. The innate immune system is formed during prenatal development and immediately responds to pathogen invasion, whereas the adaptive immune system develops during early childhood as the body continues to encounter novel pathogens and respond to specific antigens. Nevertheless, while the relationship between innate and adaptive immunity is complex, both rely on the activation of APCs.

The delivery of both adjuvant, which stimulates the immune system, and antigen, which enables a targeted immune...
response, to APCs is necessary for maximal immune activation (Figure 1). Adjuvants include pathogen-associated molecular patterns (PAMPs), characteristic molecular features recognized as foreign; and damage-associated molecular patterns (DAMPs), molecular features released from cells that are stressed or damaged. These adjuvants bind to pattern recognition receptors (PRRs) within APCs. Upon activation, APCs secrete proinflammatory cytokines and interferons to mount an innate immune response. APCs are also involved in the initiation of an adaptive immune response: they take in antigens (usually foreign proteins) and process them into shorter peptides. Antigen peptides with specific immunogenic sequences (e.g., tumor-associated or tumorspecific), are then presented on the surface of the APCs by the major histocompatibility complex (MHC) as an MHC–peptide complex. After APC maturation, the MHC–peptide complex then binds to a T cell, B cell, or other receptors (Figure 1, Signal 1). APC maturation, caused by adjuvant-activating PRRs, also leads to the upregulation of costimulatory markers that bind to the corresponding costimulation receptors on T cells (Figure 1, Signal 2). In addition, as the immune response is triggered, APCs secrete various cytokines that bind to cytokine receptors on T cells (Figure 1, Signal 3). In this way, mature APCs train cytotoxic T cells (also known as killer T cells) to rapidly multiply and destroy cancer cells that express the same antigens. Maximal T cell activation and proliferation require the engagement of a combination of these three signals initiated by APCs, suggesting the importance of delivering both adjuvants and antigens to these cells.

The choice of both adjuvant and antigen is important for vaccine design, in general, and cancer vaccine design, in particular. With respect to adjuvant selection, the most commonly employed DAMPs are aluminum salts (alum). Some of the most widely used PAMPs are unmethylated DNA strands containing multiple cytosine-guanine repeats (CpG) and bacterial polysaccharides. The most common antigens are immunogenic peptides (vide supra), although tumor-associated carbohydrate antigens (TACAs) are becoming increasingly utilized in the design of cancer vaccines as aberrant glycosylation is a hallmark of many cancers.

Vaccine development has evolved significantly from when antigen and adjuvant materials were simply mixed and coadministered as a bolus injection. In the case of cancer vaccines, their composition has changed over time as immunologists have gained mechanistic insights into the immune cascade and have identified new targets and ligands that activate immune cells against cancer. Among the various formulations, antigenic protein and adjuvant nucleic acid systems are a widely explored combination, and the identification and isolation of the most immunogenic portions of these have allowed for the development of vaccines with higher drug-to-carrier ratios that elicit fewer nonspecific immune responses than those containing larger proteins and nucleic acids. Due to advances in solid-phase chemical synthesis, peptides and oligonucleotides can now be precisely synthesized in large quantities. In addition, overall vaccine efficacy can be enhanced when nanomaterials are used to deliver immunotherapeutic agents. The incorporation of adjuvants and antigens into a nanoparticle platform protects them from premature biodegradation and increases adjuvant/antigen codelivery to particular immune cells, thus reducing systemic toxicity and enhancing the targeted immune response.

The application of chemical design and nanoscience principles to cancer immunotherapy has led to the rapid clinical development of many nanoscale vaccines. Indeed, scientific and technological advances that span multiple disciplines have paved the way for the development of next-generation nanoscale cancer vaccines. Nanoscale structural, chemical, and compositional design allows researchers to systematically synthesize cancer immunotherapeutics that impart high protective immunity with limited off-target effects; this systematic development process is termed “rational vaccinology.” A wide range of nanoparticles have been used as carriers for antigens and adjuvants, and the effects of nanoparticle size, shape, and charge on biodistribution and cellular uptake have been investigated. These studies have illuminated the fact that the precise spatiotemporal control of
the codelivery of antigens and adjuvants is critical to successfully mobilizing the immune system against a desired target. With new immunotherapeutic agents being continuously developed, it is crucial for biologists and immunologists to engage with chemists, materials scientists, and engineers in the collaborative, coordinated development of universal methods to engineer potent immunotherapies. The relative importance of each new parameter in a library of general synthetic design rules can be evaluated by engineers and data scientists using analytical high-throughput screening techniques and machine learning tools. In this Outlook, we present recent advances in vaccine design via rational vaccinology and discuss how transdisciplinary research is necessary to prepare novel vaccines in this context, primarily demonstrated by spherical nucleic acids (SNAs) as a model system.

2. NANOSCALE DESIGN TO ENHANCE INNATE IMMUNE RESPONSES

Innate immunity is categorized by the rapid response of certain classes of immune cells upon the detection of danger signals. These responses are triggered by the activation of PRRs, which include toll-like receptors (TLRs), C-type lectins (CLRs), retinoic acid inducible gene-I (RIG-I), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytosolic nucleic acid receptors. Among the various PRRs, TLRs were the first to be identified, and their importance in innate immune response was recognized by the 2011 Nobel Prize in Physiology and Medicine. As such, TLRs are one of the most widely studied and utilized PRRs for innate immune activation. Adjuvants function as danger signals to induce an innate immune response. Upon activation of TLRs by adjuvants, APCs express costimulatory markers (Signal 2) and secrete cytokines (Signal 3) that are required for T cell activation (see Figure 1). Many TLRs are located in the endosome (TLR3, TLR7/8, and TLR9) and are activated by nucleic acid (double-stranded RNA, single-stranded RNA, and single-stranded DNA, respectively) or small molecule adjuvants. Endosomal TLR activation triggers strong immune responses, and since many nanoparticles enter cells via endocytosis, delivering immunogenic nucleic acids using nanoparticles has become a popular and successful strategy. It has been shown that systems in which nucleic acid adjuvants are delivered to cells by nanoparticles significantly outperform those where the fully soluble, linear nucleic acids are administered. Nanoparticles deliver multiple copies of nucleic acids to the endosomes at once, due to their multivalent structure. This results in a high accumulation of nucleic acids in the endosome, thus promoting receptor clustering and inducing strong immune signaling. The innate

Figure 2. Examples of nanovaccine design parameters. (A) Innate immune responses, dictated by adjuvant processing, can be tuned by nanovaccine structure (encapsulated or surface-presented), ligand anchoring chemistry, and composition and identity of the pattern recognition receptor (PRR) ligand(s). (B) Adaptive immune responses, dictated by antigen processing, can be tuned by nanovaccine structure (encapsulated or surface-presented), chemistry linking the antigen to the nanovaccine, and the composition and identity of the antigen(s). SNAs are used as a model vaccine to illustrate these dependencies.
The immune response is affected by the structure, conjugation chemistry, and ligand composition of a nanovaccine (Figure 2A), and here, we primarily use spherical nucleic acid nanostructures as a model system to illustrate this point with other systems also being briefly discussed.

**2.1. Structure.** When designing nanoconstructs for the delivery of adjuvants to stimulate TLRs and an innate immune response, a key consideration is the way in which the TLR agonists are presented within the nanoscale structure (Figure 2, top left). Nucleic acid adjuvants can be encapsulated in the core of nanoparticles to prevent their premature degradation by nucleases, but nuclelease stability can also be improved when oligonucleotide TLR agonists are densely functionalized on the surface of nanoparticles in a radial orientation in the SNA form.54,55 When oligonucleotides are presented on the surface of liposomal SNAs (LSNAs, SNAs with liposomal cores), a significantly enhanced immune response is observed compared to when the oligonucleotides are encapsulated inside the LSNAs (on a per oligonucleotide basis).54,55 This effect can be attributed to two factors: (i) SNAs are rapidly taken up by APCs through receptor-mediated endocytosis, and (ii) the presentation of oligonucleotides on the surface of SNAs provides better accessibility of the adjuvants to the TLRs inside the endosomes.

In addition, the conjugation density of the surface-presented adjuvant on the nanoparticle impacts immunostimulation. For example, SNAs with higher CpG loading densities show stronger immunostimulatory activities.55 Moreover, smaller SNAs, which have higher surface curvatures and higher loading densities per unit area, induce stronger specific immune responses and minimize unintended cytokine production.56 Higher loading densities induce stronger multivalent binding to the receptors, and the surface curvature of the construct core can affect the endosomal organization, which in turn affects the downstream immune response.57 Likewise, when various hydrophilic and hydrophobic linkers were used to graft TLR7/8 small-molecule ligands onto a polymer scaffold, it was revealed that increasing the conjugation density improved immunogenicity.58–60 The immune responses have also been toggled by size and architecture (coil vs micelle vs submicron particle) with the same conjugate components.61 Together, these studies indicate that the structural location and density of the adjuvant, as well as the size and shape of the overall nanoconstruct, impact the magnitude of the innate immune response.

It is also possible to form nanostructures composed solely of adjuvant. For example, CpG DNA has been used to synthesize structures called “nanoflowers”, providing a versatile platform for delivering immunotherapeutic agents. These CpG nanoflowers were formed via rolling circle replication (RCR), which can be utilized to produce multiple copies of a single circular CpG DNA template.62 The size of the poly-CpG nanostructure can be controlled by tuning the RCR reaction time or by incorporating positively charged, PEG-grafted polypeptides that condense the negatively charged nanostructures based on electrostatic interactions.63–65 Other self-assembled multivalent CpG DNA nanostructures, such as DNA origami,66 dendrimers,67 dendrons,68 tetrahedrons,69 polyods,70 and centipedes,70 can be taken up by cells to significantly impact cytokine secretion; however, more meticulous designs and complex syntheses may be required to successfully incorporate additional payloads (e.g., antigens) without disrupting the structural features that make them useful.

**2.2. Chemistry.** The chemical properties of TLR agonist-based nanoconstructs, especially those that affect stability, impact immunostimulation and are thus another critical design parameter. DNA is typically hydrophilic, but when it is conjugated to a lipophilic moiety at a terminus, an amphiphilic bioconjugate results, which can be inserted into the bilayer membrane of a liposome (Figure 2, middle left). For example, a range of lipophilic moieties can be conjugated to DNA for synthesizing classes of LSNAs with different stabilities. In initial efforts, LSNAs were synthesized using tocopherol-functionalized DNA.71 Then, cholesterol-functionalized DNA was used as a building block because it is easier to synthesize and purify. Later, a diacyl lipid was conjugated to DNA to further improve the anchoring stability of the strand within the LSNAs core.72 More stable SNA structures facilitated multivalent binding to receptors and thus induced a faster and stronger immune response. LSNAs formed with diacyl lipid-functionalized CpG DNA exhibited enhanced serum stability, higher cellular uptake, and stronger immunostimulation compared to LSNAs synthesized with cholesterol-modified CpG DNA.72 In this case, higher serum stability is also likely to lead to more extensive serum protein corona formation, which can enhance receptor-specific endocytosis.75,76 In addition, liposomal core stability also affects overall SNA stability, where more stable core formulations (e.g., lipids of higher phase transition temperature) lead to more robust immunostimulation and higher antitumor efficacy.75 Taken together, these results suggest that the conjugation chemistry used to attach CpG DNA adjuvants to liposomes, as well as the lipid chemistry, can be used to modulate immunostimulation. These works represent a small sampling of the ways in which chemistry can be employed to modulate the stability of adjuvant incorporation into nanoconstructs, and many other schemes exist that could be explored to further probe the relationship between nanoconstruct chemistry and immune activation.

**2.3. Composition.** Nanoconstruct design allows for multiple classes of adjuvants to be codelivered to APCs on a single structure and, moreover, for the ratio of the individual adjuvants on the structures to be tuned (Figure 2, bottom left). These features are important because different CpG DNA sequences, even if they both target TLR9, likely do not activate the innate immune system in the same way.76,77 For example, synergistic immune activation was achieved with SNAs by employing a combination of class A and class B CpG DNA, which stimulates different components of the innate immune system. Enhanced activation was achieved when both classes of CpG were combined on the same SNA with controlled stoichiometry.78 Such “sequence multiplicity” promotes the activation of both early and late endosomal TLR9 during intracellular trafficking, unlike the single-component class A or B CpG SNA structures, which only briefly activate TLR9 in one of the stages. By analyzing the intracellular trafficking kinetics of each SNA component, the sequence stoichiometry was modulated to achieve potent activation. In this example, the proportion of each class of CpG on the SNAs correlated with the amount of time for which they remained in each activating organelle (i.e., early or late endosomes).

It can also be advantageous to codeliver multiple PRR ligands to enable parallel signaling, especially if these ligands coactivate pathways that require different adaptor proteins without interference. Although both TLR3 and TLR9 can trigger the production of nuclear factor kappa-B (NF-kB) and
inflammatory cytokines, TLR3 activation signals interferon regulatory transcription factor 3 (IRF3), which produces interferon (IFN)-β, while TLR9 activation signals IRF7, which leads to the secretion of IFN-α. IFN-α and IFN-β are necessary for the maintenance of memory T cells (long-lived, antigen-specific T cells).\textsuperscript{79} Interestingly, IFN-α has also been shown to upregulate TLR3 expression.\textsuperscript{80} The coadministration of CpG DNA that activates TLR9 and polyinosinic-polycytidylic acid [poly(I:C)], a synthetic double-stranded RNA analogue that activates TLR3, has been shown to lead to synergistic immune activation in a variety of cell lines and animal models.\textsuperscript{81–85} Targeted codelivery is enhanced, and immune activation is prolonged and strengthened when nanoscale vaccines containing two components are delivered compared to that when single-component nanomaterials are used.\textsuperscript{86–88}

In summary, the structural arrangement, conjugation chemistry, and composition of PRR agonists in nanovaccine constructs like SNAs play important roles in stimulating the innate immune system. Immune responses induced by nanovaccines can be modulated by understanding how structural arrangement affects agonist binding to PRRs, how stability affects PRR binding efficiency, and how composition, especially of multiple components, enables synergistic activation. With such knowledge in hand, future directions involve rationally designing and exploring structures and conjugation chemistries in the context of multiadjuvant systems.

### 3. NANOSCALE DESIGN TO ENHANCE ADAPTIVE IMMUNE RESPONSES

Antigens are biomolecules that can be used to train the adaptive immune system to target and eliminate cancer cells, making them critically important for antitumor activity and vaccine function. Typically, antigens are peptides or proteins, and they fall into two categories in the context of tumor immunotherapy: (i) antigens that are highly overexpressed on tumor cells but that are also expressed to a lesser degree on healthy cells (tumor-associated antigens) or (ii) antigens that are expressed only on specific tumor cells (tumor-specific neoantigens).\textsuperscript{89} In order for the adaptive immune response to be triggered, antigens must be delivered to activated APCs in high concentrations. Once engulfed by an APC, the antigen gets processed and presented on either the MHC-I, activating cytotoxic CD8+ T cells, or MHC-II, which activates helper CD4+ T and B cells (Signal 1) (Figures 1 and 3A).\textsuperscript{90} T cell activation has historically been of particular interest because of its central role in the adaptive immune response,\textsuperscript{91,92} although there are a variety of other immune cells and processes in play (e.g., B cell activation).\textsuperscript{93} Regardless of identity, peptide and protein antigens are often poorly immunogenic when administered on their own because they are rapidly degraded by proteases and cleared from the body.\textsuperscript{94} In addition, both antigen presentation and costimulatory molecule expression are required for T cell activation (Figure 3A), and the timing and duration of these processes play important roles in promoting T cell activation and avoiding T cell anergy (where T cells become inactivated following prior stimulation).\textsuperscript{95}
Thus, nanoscale delivery systems have been explored for the inclusion of antigens.96,97 Researchers have primarily focused on developing nanoscale cancer vaccines that incorporate MHC-I antigens to elicit CD8+ T cell responses, which underpin the adaptive cellular immune response.98 However, the development of vaccines that elicit both cellular and humoral responses could also be bolstered. Multiple examples of nanovaccines targeting helper CD4+ T cell and B cell activation, central to humoral responses, exist. However, fewer CD4+ T cell- and B cell-based peptide epitopes have been discovered as compared to CD8+ T cell-based epitopes, presenting an important current challenge for biologists and immunologists. The treatment efficiency of B cell-based vaccines relies on their ability to elicit the expression of neutralizing antibodies against peptide epitopes.99 These epitopes might not be recognizable by CD8+ T cells to induce robust, specific antitumor immune responses because they are typically processed and presented via the MHC-II pathway. However, there is evidence indicating that introducing both T and B cell epitopes in vaccines can elicit highly focused antibody responses against tumor-specific antigens.100 Thus, inducing both CD8+ and CD4+ T cell responses by incorporating multiple tumor-associated peptide antigens in SNA structures could have great potential to stimulate both cellular and humoral antitumor immune responses.

The structure, chemistry, and composition of the antigens should be carefully considered when designing nanovaccines that trigger a robust adaptive immune response (Figure 2B).

3.1. Structure. The physical arrangement of antigens within nanoscale vaccines (Figure 2, top right) strongly influences antigen delivery and overall vaccine immunogenicity and performance. For instance, a recently developed antiviral SNA encapsulating antigen [the receptor binding domain (RBD) of the full spike protein] against SARS-CoV-2 was found to robustly activate naïve B cells in human peripheral blood mononuclear cells compared to the simple mixture due to the novel multivalent three-dimensional architecture of the SNA and the ability to optimally present both adjuvant and antigen.101 Another strategy involves presenting the antigen on the nanovaccine surface. This structural arrangement has been found to be highly immunogenic because the multivalent display of peptide epitopes on a nanoparticle surface mimics the multivalent display of protein antigens on viral surfaces.102 In the case of the SNA model system, the structural arrangement of antigens was found to have a profound impact on vaccine performance.36,103 A series of LSNAs were synthesized, containing the same number of CpG DNA and peptide antigens per construct but with the peptide in different structural arrangements (Figure 3). In these vaccines, the antigen was either encapsulated in the liposome core (SNA-E), anchored onto the surface in the same manner as the CpG DNA (SNA-A), or presented on the outermost surface through conjugation to a complementary DNA sequence that was hybridized to the surface-bound CpG DNA (SNA-H) (Figure 3B). The kinetics of antigen presentation and immunostimulation by adjuvant (as measured by costimulatory marker expression) were elucidated, and it was found that both signals were best synchronized when SNA-H was used. Of these three structures, SNA-H elicited the most potent antitumor immune response (Figure 3C) and drastically extended animal survival (Figure 3D).

This study illustrates a key aspect of rational vaccinology—that it is the structural arrangement of the vaccine components, not the identities of the components alone, that dictates overall vaccine efficacy. Rational vaccinology suggests that adjuvant nucleic acid sequences or therapeutic antigen peptides that have failed out of clinical trials may demonstrate increased potency when incorporated into a nanoscale vaccine structure. This was found to be true in the case of SNAs, where prostate-specific membrane antigen, which failed to achieve clinically desirable efficacy on its own, was found to achieve substantially improved immunogenicity when incorporated into SNA form.104 In a unique example of antigen-only vaccine design, strong immune responses were observed when ovalbumin (OVA) peptide antigens were assembled into nanofibers with surfaces that displayed the epitope.105

An alternative strategy to surface presentation is to physically encapsulate antigens in liposomes because chemical conjugation is not required, and the hollow core can be utilized;106 however, the encapsulation efficiency and nanoparticle stability may limit the overall efficacy of structures prepared using this approach. It can be difficult to control how much cargo is encapsulated and how quickly it is released from the nanoparticle core.107 Strategies that involve tailoring the chemistry of the core structures (liposome108 or multilamellar vesicles) to tune their stability and antigen release have been devised to overcome these issues. In the latter example, OVA was used as a prototypical antigen and encapsulated in multilamellar vesicles, where an inter-bilayer cross-linker was used to stabilize the construct until exposure to lipases in the endolysosome.

3.2. Chemistry. It is important to note that, when peptide structure is altered, peptide presentation and immunogenicity may be impacted;109 how the antigens are chemically conjugated to the nanovaccine, and their chemical form when they are released, can significantly affect downstream processing. Thus, the design and synthesis of the antigenic components on nanoscale vaccines should be carefully considered (Figure 2, middle right). For instance, the chemical conjugation method utilized to link the peptide antigens to the complementary DNA sequences hybridized to the surface-bound DNA in SNA-H (see Figure 3B) has a dramatic impact on antigen processing and downstream T cell response, in terms of both activation and proliferation.110 In this system, the use of a “traceless” linker resulted in the cleavage and release of the peptide antigen from the SNA scaffold in an unmodified form (i.e., with no added chemical moieties), while a “cleavable but nontraceless” linker resulted in the release of a peptide with a pendant group and a “noncleavable” linker control did not foster cleavage and release. The use of the traceless linker led to up to an 8-fold improvement in T cell proliferation compared to when SNAs with the other two types of linkers were used. Higher levels of cytokine and T cell activation were also observed with the traceless SNA formulations. Furthermore, these traceless linkers were designed with different cleavage rates to fine-tune the antigen release rates and therefore control antigen presentation kinetics.112

In another study, the processing and presentation of OVA peptides chemically conjugated to polymeric nanoparticles revealed that the use of reducible linkages led to higher antigen presentation and enhanced vaccine performance relative to when nonreducible linkages were used. These results suggest
that the use of nonreducible linkages may interfere with antigen presentation and downstream activation.\textsuperscript{113}

A potent nanoscale vaccine also was prepared that was composed of high-density lipoprotein-mimicking nanodiscs conjugated to antigen peptides through a reducible linker.\textsuperscript{114} Specifically, the OVA-derived peptide epitope SIINFEKL was covalently conjugated to the nanodiscs through a disulfide linker as well as electrostatically complexed with CpG DNA adjuvant. Improved codelivery of adjuvant and antigen to lymph nodes and sustained antigen presentation on dendritic cells was observed compared to their soluble counterparts. Similarly, experiments involving polymeric nanoparticles containing OVA protein functionalized via disulfide groups and electrostatically complexed CpG DNA show that OVA conjugation to the nanoparticle significantly increases antigen presentation relative to simple vaccine mixtures.\textsuperscript{115}

3.3. Composition. The composition and identity of antigens within nanoscale vaccines can be tailored to elicit the antigen-specific immune response that induces tumor killing (Figure 2, bottom right). Furthermore, multiple classes of peptide antigens can be delivered via the same nanoparticle to enhance the antitumor immune response.\textsuperscript{116} Cancer vaccines that target multiple tumor antigens have the potential to more efficiently target the tumor cells by the simultaneous recognition of multiple antigens. For this reason, it may be beneficial to deliver a combination of different antigens in a single construct.\textsuperscript{117} In a recent example, mesoporous silica microrod vaccines combined with poly(ethylenimine) were synthesized to contain peptide antigen pools from melanoma and colorectal carcinoma.\textsuperscript{110} The resulting structures elicited broad immune responses and decreased tumor escape.

Alternatively, proteins isolated from tumor cells, called lysates, can be utilized as the antigen source in nanoscale vaccines. The use of lysates can address the shortcomings of some peptide-based vaccines, including immune evasion caused by tumor mutation and low antigen presentation on the tumor cell surface.\textsuperscript{118–120} The immune system was potently activated against tumors when protein lysates from melanoma cells were encapsulated in porous polymeric scaffolds together with TLR agonists and chemokines (a cytokine subclass) to recruit APCs to the infection site.\textsuperscript{121} However, it is important to note that the manner in which lysates are isolated and prepared can have a dramatic impact on immunogenicity and vaccine potency.\textsuperscript{122} This feature may be attributed to the types of danger signals produced by tumor cells when subjected to different processing methods\textsuperscript{123} as well as the production of different antigens based on processing methods.\textsuperscript{124} Indeed, it was discovered that, for liposomal SNAs with encapsulated tumor cell lysates, lysates that were oxidized via exposure to hypochlorous acid were significantly more immunogenic than lysates that had not been oxidized.\textsuperscript{125} This enhanced immunogenicity is thought to be due to chemical and structural changes to the lysate antigen that occur upon oxidation, changing how it is recognized and processed within immune system pathways.\textsuperscript{126}

These examples highlight that the structural arrangements, conjugation and modification chemistry, and composition of antigens in nanoconstructs play important roles in stimulating the adaptive immune system. When these parameters are changed, so too are antigen processing and presentation kinetics and efficiency. Future directions involve the design of novel structural arrangements or combinations of multiple arrangements that allow one to tune the antigen presentation kinetics or prolong antigen presentation time. New chemical modifications of antigens for more efficient antigen processing may also be desirable. In addition, other molecular compositions that support immune function, beyond adjuvants and antigens, should be considered for integration into future nanoscale vaccines. Indeed, checkpoint inhibitor therapy has become a standard combination therapy for a broad spectrum of cancer types.\textsuperscript{127–129} In checkpoint inhibitor therapy, monoclonal antibodies are administered to block regulatory checkpoint receptors that prevent tumoricidal immune function, and such types of antibodies can be incorporated into SNAs via the protein corona.\textsuperscript{74} Cytokines have also been incorporated in vaccine designs as potential adjuvants. For example, the cytokine interleukin 2 (IL-2) was explored in a combination therapy due to its ability to regulate T cell and natural killer cell responses.\textsuperscript{130} Furthermore, chemokines, such as granulocyte-macrophage colony-stimulating factor, that

\textbf{Figure 4.} Scalable rational vaccinology enabled by high-throughput screening and data science. (A) Schematic illustration of the high-throughput screening of nanoscale vaccines by SAMDI. (B) High-throughput screening data can be processed using supervised machine learning and can predict quantitative structure–activity relationships with high accuracy (modified from ref 135).
draw immune cells to tumor sites have also been investigated as vaccine components due to their ability to regulate dendritic cell development and subsequent T cell activation through the management of costimulatory factors. However, it remains a challenge to determine the ideal dose for optimal immune activation, and this issue has hindered clinical translation.131 These aspects may benefit from high-throughput screening techniques.

4. SCREENING AND PREDICTING THE DESIGN SPACE

As presented above, there are many design parameters related to the chemistry and structures of nanomedicines that can affect immune response and efficacy. With such a vast design space, it is challenging to directly evaluate combinations of effects by picking and choosing parameters from across different nanoparticle systems.132 However, researchers can take advantage of high-throughput screening techniques to probe the combinatorial design space, and this is one area where it is important for chemists and materials scientists to interface with computational chemists and data scientists. Self-assembled monolayers for matrix-assisted laser desorption/ionization (SAMDI) mass spectrometry is a useful tool in this context.133,134 SAMDI uses a high-throughput mass spectrometry plate that is coated with a self-assembled monolayer presenting a moiety to selectively capture analytes and then quantify the analyte concentration. In a pilot study, high-throughput screening with SAMDI was used to test the design space of a library of 960 SNAs where 11 independently controlled properties of the nanoparticle core, antigen, and adjuvant were varied, and their effects on immune activation were studied.135 After treating cells with SNAs, SAMDI was used to measure cytokine production and secreted alkaline phosphatase present in the cell culture supernatant, indicating the level of immune activation (Figure 4A).136 The data were analyzed using statistical models to identify leading effectors and nonlinear mixed effects between different parameters. It was found that conjugation chemistry has an exceptionally strong effect on immune activation—this and other findings from this study were used to establish design rules for achieving maximally potent SNAs.135 Given the vast multidimensional space, these data only represent a small subset of the samples that can be produced. However, the data can be used for supervised machine learning via structure–activity relationship analysis to predict the performance of untested variants with high statistical confidence (Figure 4B).135 With new antigens being identified and new material structures being designed, such a process will facilitate a rapid understanding of the structure–activity relationships of nanomedicines without the need to test the entire library of possible designs. Indeed, when the data from these libraries were used to train the model, it was found that only a small subset of structures (16%) was needed to accurately make predictions. The results of these studies can be used to identify the properties (or nonobvious or nonlinear combinations of properties) that are most important for maximizing immune activation in high throughput, guiding future studies toward the properties that have the greatest impact on performance.

Although some vaccine design parameters (e.g., size and concentration) can be regarded as continuous variables in the context of data science and machine learning, most of the design parameters presented herein (e.g., structure, chemistry, and composition) are non-numerical and therefore discrete in nature. It may be challenging to use traditional supervised machine learning to predict the design space because these discrete parameters are difficult to directly incorporate into regression models. Therefore, deep learning techniques may be needed to obtain the quantitative structure–activity relationship for new nanoscale vaccine designs and predict their potential effects on efficacy. These findings and models should also be combined with clinical pharmacology to predict clinical performance and perform clinical trials most efficiently, bringing clinicians into the fold. An in vitro–in vivo performance correlation is important, but a valid in vitro–clinical performance correlation is arguably more important. Therefore, it is crucial to establish a set of quantitatively stable design rules for nanoscale vaccines, perhaps by also incorporating patient conditions as parameters to produce personalized nanomedicines that could work most effectively for an individual. It is important to note that data mining techniques to search for similar sequences and predict their therapeutic efficacy in nanoscale vaccine designs have the potential to substantially improve vaccine development.

5. CONCLUSIONS

In summary, the importance of rational vaccinology in SNA vaccine development, and cancer vaccine development more broadly, is clear: besides the identity of individual vaccine components, how and when they are presented in the delivery system are also critically important for immunotherapeutic efficacy. Even if the components are carefully discovered for specific targets, the incorrect structural arrangement of these components may lead to inferior performance. Therefore, experts from a range of disciplines are needed to fully realize the potential of rational vaccinology-based approaches to immunotherapy. Biologists and immunologists are needed to elucidate the signaling pathways and identify new immunotherapeutic agents. Chemists can design and synthesize chemical modifications of immunotherapeutic agents and develop novel conjugation chemistries. Materials scientists can design nanomaterials with properties that are more favorable for immunotherapeutic efficacy. Engineers can identify novel structural and compositional design spaces for incorporating various immunotherapeutic agents into the nanoscale vaccine systems. Finally, data scientists can analyze the design space and identify key parameters for rational vaccine designs and guide researchers in other fields. Researchers with backgrounds that are traditionally unrelated to immunotherapy can widely contribute their expertise toward advances in rational vaccinology at the nanoscale. This collaborative approach to rational vaccinology recently culminated in human clinical trials in which CpG adjuvant-carrying TLR9-agonistic SNAs were administered to patients alone and in combination with immune checkpoint inhibitors (NCT03086278; NCT03684785).137,138 This SNA drug shrunk tumor targets in 37% of patients with a confirmed overall response rate of 33% at the highest selected Phase 2 dose for Merkel cell carcinoma (MCC).139

Moving forward, chemists and other researchers should continue to use rational vaccinology to elucidate the fundamental principles necessary for the development of cancer vaccines that are both highly immunogenic and highly tolerable. This effort will be enabled by the advanced understanding of how structure impacts function (e.g., targeting (or multitargeting) to and retention in specific cell types or subcellular spaces, temporal control and component release, delivery of large materials, produg
behavior, and toxicity] and how the arrangement of vaccine components can be tuned at the nanoscale to afford various maximal immune responses. With design rules established and key design parameters identified, scientists can significantly broaden the impact of immunotherapies by taking advantage of tools from chemistry, biology, materials science, engineering, and data science to find the formulations of the most potent immunotherapeutics. This knowledge, along with the toolkit developed to date in the context of SNAs and other materials, will allow for the rapid design, synthesis, testing, and deployment of nanovaccines against diverse cancer types and the application of the lessons learned to the treatment of novel bacterial and viral pathogens and autoimmune and other diseases. Indeed, of the infinite number of nanostructures that can be prepared, we can use the prototypical SNA system as a guide, and chemical knowledge described herein can be used to build other structures if a similar structure-focused protocol is adopted. In fact, if one follows this supposition to a logical end point, one concludes that the ultimate vaccine structures will be modular, nanoscale in size, and molecularly pure where the position and connectivity of every atom has been optimized for signaling control and therapeutic efficacy. This is where molecularly precise entities like peptide-functionalized dendrimers may play a significant role.139,140

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Awards R01CA208783 and P50CA221747. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This material is also based upon work supported by the Polsky Urologic Cancer Institute of the Robert H. Lurie Comprehensive Cancer Center of Northwestern University at Northwestern Memorial Hospital. C.E.C. was supported by a Postdoctoral Fellowship, PF-20-046-01-LIB, from the American Cancer Society, as well as the Eden and Steven Romick Postdoctoral Fellowship through the American Committee for the Weizmann Institute of Science. M.E. was supported by the Alexander S. Onassis Public Benefit Foundation.

■ REFERENCES

(1) Kelly, P. N. The Cancer Immunotherapy Revolution. Science 2018, 359, 1344–1345.

(2) Lin, A.; Giuliano, C. J.; Palladino, A.; John, K. M.; Abramowicz, C.; Yuan, M. L.; Sausville, E. L.; Lukow, D. A.; Liu, L.; Chait, A. R.; Galluzzo, Z. C.; Tucker, C.; Sheltzer, J. M. Off-Target Toxicity Is A Common Mechanism of Action of Cancer Drugs Undergoing Clinical Trials. Sci. Transl. Med. 2019, 11, eaaw8412.

(3) Cancer Multidrug Resistance. Nat. Biotechnol. 2000, 18, IT18–IT20.

(4) Seyfried, T. N.; Huysentruyt, L. C. On the Origin of Cancer Metastasis. 2013, 18, 43–73.

(5) Milling, L.; Zhang, Y.; Irvine, D. J. Delivering Safer Immunotherapies for Cancer. Adv. Drug Delivery Rev. 2017, 114, 79–101.

(6) Hopton Cann, S. A.; van Netten, J. P.; van Netten, C. Dr William Coley and Tumour Regression: A Place in History or in the Future. Postgrad Med. J. 2003, 79 (938), 672–680.

(7) Park, J.-E.; Jardine, L.; Gottgens, B.; Teichmann, S. A.; Hanifia, M. Prenatal Development of Human Immunity. Science 2020, 368, 600–603.

(8) Kollmann, T. R.; Marchant, A.; Way, S. S. Vaccination Strategies to Enhance Immunity in Neonates. Science 2020, 368, 612–615.

(9) Lewis, S. M.; Williams, A.; Eisenbarth, S. C. Structure and Function of the Immune System in the Spleen. Science Immunology 2019, 4, eaau6085.

(10) Iwasaki, A.; Medzhitov, R. Regulation of Adaptive Immunity by the Innate Immune System. Science 2010, 327, 291–295.

(11) De Temmerman, M.-L.; Rejman, J.; Demeester, J.; Irvine, D. J.; Gander, B.; De Smedt, S. C. Particulate Vaccines: On the Quest for Optimal Delivery and Immune Response. Drug Discovery Today 2011, 16, 569–582.

(12) Sun, H.; Hu, W.; Yan, Y.; Zhang, Z.; Chen, Y.; Yao, X.; Teng, L.; Wang, X.; Chai, D.; Zheng, J. Using Pamps and Damps as Adjuvants in Cancer Vaccines. Human Vaccines & Immunotherapeutics 2021, 17, 5546.

(13) Svensson, A.; Sandberg, T.; Siesjö, P.; Eriksson, H. Sequestering of Damage-Associated Molecular Patterns (Damps): A Possible Mechanism Affecting the Immune-Stimulating Properties of Aluminium Adjuvants. Immunol. Res. 2017, 65, 1164–1175.

(14) Amarante-Mendes, G. P.; Adjemian, S.; Branco, L. M.; Zanetti, L. C.; Weinlich, R.; Bortoluci, K. R. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. Front Immunol. 2018, 9, 2379.

(15) Vigneron, N.; Stroobant, V.; Chapio, J.; Ooms, A.; Degiovanni, G.; Morel, S.; van der Bruggen, P.; Boon, T.; Van den Eynde, B. J. An
Antigenic Peptide Produced by Peptide Splicing in the Proteasome. Science 2004, 304, 587–590.

(16) Neefjes, J.; Ovaa, H. A. Peptide’s Perspective on Antigen Presentation to the Immune System. Nat. Chem. Biol. 2013, 9, 769–775.

(17) Neefjes, J.; Jongsm, M. L. M.; Paul, P.; Bakke, O. Towards a Systems Understanding of Mhc Class I and Mhc Class II Antigen Presentation. Nat. Rev. Immunol 2011, 11, 823–836.

(18) Esensten, J. H.; Helou, Y. A.; Chopra, G.; Weiss, A.; Bluestone, J. A. CD28 Costimulation: From Mechanism to Therapy. Immunity 2016, 44, 973–988.

(19) Altam-Bonnet, G.; Mukherjee, R. Cytokine-Mediated Communication: A Quantitative Appraisal of Immune Complexity. Nat. Rev. Immunol 2019, 19, 205–217.

(20) Marchingo, J. M.; Kan, A.; Sutherland, R. M.; Dufy, K. B.; Wellard, C. J.; Belz, G. T.; Lew, A. M.; Dowling, M. R.; Heinzl, S.; Hodgkin, P. D. Antigen Affinity, Costimulation, and Cytokine Inputs Sum Linearly to Amplify T Cell Expansion. Science 2014, 346, 1123–1127.

(21) Powell, B. S.; Andrianov, A. K.; Fusco, P. C. Polyion Vaccine Adjuvants: Another Look at Aluminum Salts and Polyelectrolytes. Clinical and experimental vaccine research 2015, 4, 23–45.

(22) Vollmer, J.; Weeratna, R.; Poyette, P.; Jurk, M.; Schetter, C.; Laucht, M.; Wader, T.; Thul, S.; Liu, M.; Davis, H. L.; Krieg, A. M. Characterization of Three CpG Oligodeoxynucleotide Classes with Distinct Immunostimulatory Activities. Eur. J. Immunol. 2004, 34, 251–262.

(23) Sun, B.; Yu, S.; Zhao, D.; Guo, S.; Wang, X.; Zhao, K. Polysaccharides as Vaccine Adjuvants. Vaccine 2018, 36, 5226–5234.

(24) Jin, K.-T.; Lan, H.-B.; Chen, X.-Y.; Wang, S.-B.; Ying, X.-J.; Lin, Y.; Mou, X.-Z. Recent Advances in Carbohydrate-Based Cancer Vaccines. Biotechnol. Lett. 2019, 41, 641–650.

(25) Peixoto, A.; Relvas-Santos, M.; Azevedo, R.; Santos, L. L.; Ferreira, J. A. Protein Glycosylation and Tumor Microenvironment Alterations Driving Cancer Hallmarks. Frontiers in Oncology 2019, 9, 380.

(26) Moyer, T. J.; Zmolek, A. C.; Irvine, D. J. Beyond Antigens and Adjuvants: Formulating Future Vaccines. J. Clin Investig 2016, 126, 799–808.

(27) Hubbell, J. A.; Thomas, S. N.; Swartz, M. A. Materials Engineering for Immunomodulation. Nature 2009, 462, 449–460.

(28) Wang, Z.-B.; Xu, J. Better Adjuvants for Better Vaccines: Progress in Adjuvant Delivery Systems, Modifications, and Adjuvant–Antigen Codelivery. Vaccines 2020, 8, 128.

(29) Purcell, A. W.; McCluskey, J.; Rossjohn, J. More Than One Reason to Rethink the Use of Peptides in Vaccine Design. Nat. Rev. Drug Discovery 2007, 6, 404–414.

(30) Krieg, A. M. CpG Motifs: The Active Ingredient in Bacterial Nucleic Acid-Sensing TLRs: Trafficking and Regulation. Curr. Opin Immunol 2017, 44, 26–33.

(31) Choi, C. H.; Hao, L.; Narayan, S. P.; Auyeung, E.; Mirkin, C. A. Mechanism for the Endocytosis of Spherical Nucleic Acid Nanoparticles. Proc. Natl. Acad. Sci. U. S. A. 2013, 110, 7625–7630.

(32) Radovic-Moreno, A. F.; Chernyak, N.; Mader, C. C.; Dallapatas, S.; Kang, R. S.; Hao, L.; Walker, D. A.; Halo, T. L.; Merkel, T. J.; Riche, C. H.; Anantatmula, S.; Burkhart, M.; Mirkin, C. A.; Gryaznov, S. M. Immunomodulatory Spherical Nucleic Acids. Proc. Natl. Acad. Sci. U. S. A. 2015, 112, 3892–3897.

(33) de Titta, A.; Ballester, M.; Julier, Z.; Nembrini, C.; Jeanbart, L.; van der Vlies, A. J.; Swartz, M. A.; Hubbell, J. A. Nanoparticle Conjugation of CpG Enhances Adjuvancy for Cellular Immunity and Memory Recall at Low Dose. Proc. Natl. Acad. Sci. U. S. A. 2013, 110, 19902–19907.

(34) Dane, E. L.; Irvine, D. J. Big Thinking for Adjuvants. Nat. Biotechnol. 2015, 33, 1146–1148.

(35) Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. A DNA-Based Method for Rationally Assembling Nanoparticles into Macroscopic Materials. Nature 1996, 382, 607–609.

(36) Cutler, J. I.; Auyeung, E.; Mirkin, C. A. Spherical Nucleic Acids. ACS Cent. Sci. 2012, 134, 1376–1391.

(37) Guan, C.; Chernyak, N.; Domínguez, D.; Cole, L.; Zhang, B.; Mirkin, C. A. RNA-Based Immunostimulatory Liposomal Spherical Nucleic Acids as Potent TLR7/8 Modulators. Small 2018, 14, e1803284.

(38) Pallares, R. M.; Choo, P.; Cole, L. E.; Mirkin, C. A.; Lee, A.; Odom, T. W. Manipulating Immune Activation of Macrophages by Tuning the Oligonucleotide Composition of Gold Nanoparticles. Biomacromolecules 2019, 30, 2032–2037.

(39) Yu, J.; Pallares, R. M.; Cole, L. E.; Coughlin, E. E.; Mirkin, C. A.; Lee, A.; Odom, T. W. Smaller CpG-Conjugated Gold Nanoconstructs Achieve Higher Targeting Specificity of Immune Activation. ACS Appl. Mater. Interfaces 2018, 10, 21920–21926.

(40) Lee, K.; Huang, Z. N.; Mirkin, C. A.; Odom, T. W. Endosomal Organization of CpG Constructs Correlates with Enhanced Immune Activation. Nano Lett. 2020, 20, 6170–6175.

(41) Lynn, G. M.; Laga, R.; Darrah, P. A.; Ishizuka, A. S.; Balaci, A.; Ducley, A. E.; Pechar, M.; Pala, R.; Gerner, M. Y.; Yamamoto, A.; Buechler, C. R.; Quinn, K. M.; Smelkinson, M. G.; Vaneck, O.;
Cawood, R.; Hills, T.; Vasilatyi, O.; Kastenmüller, K.; Francica, J. R.; Stutts, L.; et al. In Vivo Characterization of the Physicochemical Properties of Polymer-Linked TLR Agonists That Enhance Vaccine Immunogenicity. Nat. Biotechnol. 2015, 33, 1201–1210.

(59) Baharom, F.; Ramirez-Valdez, R. A.; Tobin, K. K. S.; Yamane, H.; Dutertre, C.-A.; Khalilnezhad, A.; Reynoso, G. V.; Coble, V. L.; Lynn, G. M.; Mulé, M. P.; Martins, A. J.; Finnigan, J. P.; Zhang, X. M.; Hamerman, J. A.; Bhardwaj, N.; Tsang, J. S.; Hickman, H. D.; Ginhoux, F.; Ishizuka, A. S.; Seder, R. A. Intravenous Nanoparticle Vaccination Generates Stem-Like Tcf1+ Neoantigen-Specific CD8+ T Cells. Nat. Immunol. 2021, 22, 41–52.

(60) Lynn, G. M.; Seddik, C.; Baharom, F.; Zhu, Y.; Ramirez-Valdez, R. A.; Coble, V. L.; Tobin, K., Nichols, S. R.; Itzkowitz, Y.; Zaidi, N.; Gammon, J. M.; Blobel, N. J.; Denizeau, J.; de la Rochere, P.; Francica, B. J.; Decker, B.; Maciejewski, M.; Cheung, J.; Yamane, H.; Smelkinson, M. G.; et al. Peptide–TLR-7/8 Conjugate Vaccines Chemically Programmed for Nanoparticle Self-Assembly Enhance CDS T-Cell Immunity to Tumor Antigens. Nat. Biotechnol. 2020, 38, 320–332.

(61) Lynn, G. M.; Chytli, P.; Francica, J. R.; Lagová, A.; Kueberuwa, B.; Mirkin, C. A. Sequence Multiplicity within Spherical Nucleic Acids. Nucleic Acids Res. 2012, 40, 2181–2192.

(62) Zhang, L.; Zhu, G.; Mei, L.; Wu, C.; Qiu, L.; Cui, C.; Liu, Y.; Teng, I. T.; Tan, Y. W. Self-Assembled DNA Immunoflowers as Multivalent CpG Nonaognists. ACS Appl. Mater. Interfaces 2015, 7, 24069–24074.

(63) Zhu, G.; Liu, Y.; Yang, X.; Kim, Y.-H.; Zhang, H.; Jia, R.; Liao, H.-S.; Jin, A.; Lin, J.; Aronova, M.; Leapman, R.; Nie, Z.; Niu, G.; Chen, X. DNA–Inorganic Hybrid Nanovaccine for Cancer Immunotherapy. Nano Lett. 2016, 16, 6684–6692.

(64) Zhu, G.; Mei, L.; Vishwasrao, H. D.; Jacobson, O.; Wang, Z.; Liu, Y.; Yung, B. C.; Fu, X.; Jin, A.; Niu, G.; Wang, Q.; Zhang, F.; Shroff, H.; Chen, X. Intertwining DNA–RNA Nanocapsules Loaded with Tumor Neoantigens as Synergistic Nanovaccines for Cancer Immunotherapy. Nat. Commun. 2017, 8, 1482.

(65) Ni, Q.; Zhang, F.; Liu, Y.; Wang, Z.; Yu, G.; Liang, B.; Niu, G.; Su, T.; Zhu, G.; Lu, G.; Zhang, L.; Chen, X. A Bi-Adjuvant Nanovaccine That Potentiates Immunogenicity of Neoantigen for Combination Immunotherapeutic Colorectal Cancer. Sci. Adv. 2020, 6, eaaz4371.

(66) Schüller, V. J.; Heidegger, S.; Sandholzer, N.; Nickels, P. C.; Suhartha, N. A.; Endres, S.; Bourquin, C.; Liedl, T. Cellular Immunostimulation by CpG-Sequence-Coated DNA Origami Structures. ACS Nano 2011, 5, 9696–9702.

(67) Li, J.; Pei, H.; Zhu, B.; Liang, L.; Wei, M.; He, Y.; Chen, N.; Li, D.; Huang, Q.; Fan, C. Self-Assembled Multivalent DNA Nanostuctures for Noninvasive Intracellular Delivery of Immunostimulatory CpG Oligonucleotides. ACS Nano 2011, 5, 8783–8789.

(68) Mohri, K.; Kusuki, E.; Ohtsuki, S.; Takahashi, N.; Endo, M.; Hidaka, K.; Sugiyama, H.; Takahashi, Y.; Takakura, Y.; Nishikawa, M. Self-Assembling DNA Dendrimer for Effective Delivery of Immunostimulatory CpG DNA to Immune Cells. Biomacromolecules 2015, 16, 1095–1101.

(69) Mohri, K.; Nishikawa, M.; Takahashi, N.; Shiomi, T.; Matsuoka, N.; Ogawa, K.; Endo, M.; Hidaka, K.; Sugiyama, H.; Takahashi, Y.; Takakura, Y.; Nishikawa, M. Design and Development of Nanopized DNA Assemblies in Polypod-Like Structures as Efficient Vehicles for Immunostimulatory CpG Motifs to Immune Cells. ACS Nano 2012, 6, 5931–5940.

(70) Li, W.; Luo, L.; Huang, J.; Wang, Q.; Liu, J.; Xiao, X.; Fang, H.; Yang, X.; Wang, K. Self-Assembled DNA Nanocipettes as Multivalent Vehicles for Enhanced Delivery of CpG Oligonucleotides. Chem. Commun. 2017, 53, 5565–5568.

(71) Banga, R. J.; Chernyak, N.; Narayan, S. P.; Nguyen, S. T.; Mirkin, C. A. Liposomal Spherical Nucleic Acids. J. Am. Chem. Soc. 2014, 136, 9866–9869.
J.; Horn, L.; Drake, C. G.; Pardoll, D. M.; Chen, L.; Sharfman, W. H.;
Anders, R. A.; Taube, J. M.; et al. Safety, Activity, and Immune
Correlates of Anti-Pd-1 Antibody in Cancer. *N Engl J. Med.* 2012,
366, 2443–2454.

(128) Zou, W.; Wolchok, J. D.; Chen, L. Pd-L1 (B7-H1) and Pd-1
Pathway Blockade for Cancer Therapy: Mechanisms, Response
Biomarkers, and Combinations. *Sci. Transl Med.* 2016, 8, 328rv324.

(129) Topalian, S. L.; Taube, J. M.; Pardoll, D. M. Neoadjuvant
Checkpoint Blockade for Cancer Immunotherapy. *Science* 2020,
367, eaax0182.

(130) Sondel, P. M.; Hank, J. A. Combination Therapy with
Interleukin-2 and Antitumor Monoclonal Antibodies. *Cancer Journal
from Scientific American* 1997, 3, S121–S127.

(131) Zhao, W.; Zhao, G.; Wang, B. Revisiting Gm-Csf as an
Adjuvant for Therapeutic Vaccines. *Cell Mol. Immunol* 2018, 15, 187–
189.

(132) Liu, Y.; Chen, X. Efficient Screening of Spherical Nucleic
Acids. *Nature Biomed Eng.* 2019, 3, 257–258.

(133) Mrksich, M. Mass Spectrometry of Self-Assembled Mono-
layers: A New Tool for Molecular Surface Science. *ACS Nano* 2008, 2,
7–18.

(134) Min, D.-H.; Tang, W.-J.; Mrksich, M. Chemical Screening by
Mass Spectrometry to Identify Inhibitors of Anthrax Lethal Factor.
*Nat. Biotechnol.* 2004, 22, 717–723.

(135) Yaman Kurt, G.; Berns, E. J.; Xue, A.; Lee, A.; Bagheri, N.;
Mrksich, M.; Mirkin, C. A. Exploration of the Nanomedicine-Design
Space with High-Throughput Screening and Machine Learning. *Nat.
Biomed Eng.* 2019, 3, 318–327.

(136) Berns, E. J.; Cabezas, M. D.; Mrksich, M. Cellular Assays with
a Molecular Endpoint Measured by Samdi Mass Spectrometry. *Small*
2016, 12, 3811–3818.

(137) Exicure, Inc. *A Study of Ast-008 in Healthy Subjects*, 2017,
NCT03086278. https://clinicaltrials.gov/ct2/show/NCT03086278.

(138) Exicure, Inc. *Intratumoral Cavrotolimod Combined with
Pembrolizumab or Cemiplimab in Patients with Merkel Cell Carcinoma,
Cutaneous Squamous Cell Carcinoma, or Other Advanced Solid Tumors*,
2018, NCT03684785. https://clinicaltrials.gov/ct2/show/
NCT03684785.

(139) Distler, M. E.; Teplensky, M. H.; Bujold, K. E.; Kusmierz, C.
D.; Evangelopoulos, M.; Mirkin, C. A. DNA Dendrons as Agents for
Intracellular Delivery. *J. Am. Chem. Soc.* 2021, 143, 13513–13518.

(140) Cheng, H. F.; Distler, M. E.; Lee, B.; Zhou, W.; Weigand, S.;
Mirkin, C. A. Nanoparticle Superlattices through Template-Encoded
DNA Dendrimers. *J. Am. Chem. Soc.* 2021, 143, 17170–17179.