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Nucleic Acid Nanoparticles (NANPs) as Molecular Tools to Direct Desirable and Avoid Undesirable Immunological Effects

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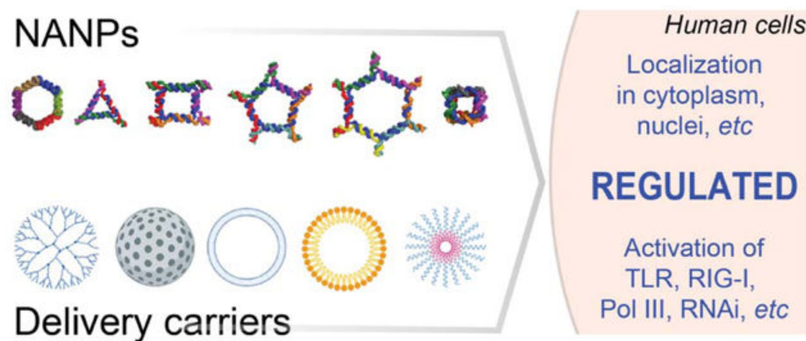
Abstract

Nucleic acid nanoparticles (NANPs) represent a highly versatile molecular platform for the targeted delivery of various therapeutics. However, despite their promise, further clinical translation of this innovative technology can be hindered by immunological off-target effects. All human cells are equipped with an arsenal of receptors that recognize molecular patterns specific to foreign nucleic acids and understanding the rules that guide this recognition offer the key rationale for the development of therapeutic NANPs with tunable immune stimulation. Numerous recent studies have provided increasing evidence that in addition to NANPs' physicochemical properties and therapeutic effects, their interactions with cells of the immune system can be regulated through multiple independently programmable architectural parameters. The results further suggest that defined immunomodulation by NANPs can either support their immunoquiescent delivery or be used for conditional stimulation of beneficial immunological responses.

Graphical Abstract



Combinations of different Nucleic Acid Nanoparticles (NANPs) and delivery agents allows to direct desirable and avoid undesirable immunological effects and therapeutic actions.

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Versatile Therapeutic Nucleic Acids (TNAs).

In the makeup of a traditional small molecule drug, the dianophore, which is the molecular component determining distribution and delivery, and the pharmacophore, which is the molecular component determining targeted function, are separate activities which are both determined by the drug's overall chemical structure. Any changes to the makeup in the drug design process can therefore mean for divergent effects between the dianophore and pharmacophore activities, which must be clinically reevaluated for every stage of the drug's development[1]. Instead, modular therapeutic nucleic acids (TNAs) offer a means of separation between these two facets, because the backbone chemistry and targeting moieties which serve as the dianophore can be used in combination with different nucleotide sequences serving as the pharmacophores with functional independence[1, 2]. Therefore, clinical evaluations of TNAs contribute to a foundation of preliminary bioactivity not only for the individual TNA candidate, but for any TNA with that modular piece in its formulation. Overall, this information and enhanced prediction of biological activity serves to greatly increase the safety profiles of TNA formulations and also has the potential to decrease the overall costs associated with the broad amount of drug candidates entering the clinical pipeline. With 156 TNAs currently undergoing phases I-III clinical trials or awaiting regulatory decisions as of April 2020, this innovative class of therapeutics holds great promise for the regulation of cells to eliminate specific diseases or enhance responses such as for cancer immunotherapy[3, 4].

With the decoding of the human genome and increasing utility in high-throughput sequencing, there has been a growing potential of TNAs to be sequence-specific candidates for targeted gene therapy[5]. A number of TNAs which vary in mechanism have been approved by the U.S. Food and Drug Administration (FDA), including antisense oligonucleotides (ASOs), mRNAs, siRNAs, miRNAs, and aptamers[6] (Fig. 1). ASOs are short sequences designed to bind to specific RNAs to promote their degradation, cleavage, or steric blocking [7]. To date, there are six antisense oligonucleotides which have been approved by the U.S. FDA: three RNase H-competent ASOs (Fomivirsen, Mipomersen, and Inotersen) and three splice-switching ASOs (Eteplirsen, Golodirsen, and Nusinersen)[6]. Two siRNA candidates (Patisiran and Givosiran), which work by undergoing enzyme-mediated RNA interference for post-transcriptional gene silencning, have recently been FDA-approved. There is also one aptamer (Pegaptanib) currently approved[6]. Aptamers are oligonucleotides selected in vitro to specifically recognize and bind a target molecule with high specificity—in this case, as an agonist to prevent angiogenesis[8]. Recently, the versatility of a nucleic acid-based approach has been further expanded by the rapid development and ongoing worldwide distribution of mRNA-based vaccines (BNT162b2 and mRNA-1273) against SARS-CoV-2[9].

Immunorecognition of TNAs.

Currently available TNAs are often offered as periodic doses, perform a single function, and may present a high recurring cost to patients[10]. The scaled-up production of nucleic acids while maintaining high purity is a challenge[11]. Furthermore, many TNAs require chemical

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modifications in order to maintain nuclease degradation resistance, cross the plasma membrane, and avoid unwanted side effects from off-target immunostimulation[12]. Pattern recognition receptors (PRRs) are strategically localized to the endosome and cytosol for the targeted recognition of pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) which allow for the discrimination of self- and non-self-biomaterials. These PRRs include the endosomal Toll-Like Receptors (TLRs)—TLR3, TLR7, TLR8, and TLR9—and the cytosolic PRRs, such as RIG-I-like receptors (RLRs) and DNA sensors. Importantly, activation of these PRRs stimulates downstream signaling cascades that trigger the production of immune mediators. The immune mediators promote cellular recruitment, cellular maturation, and antigen presentation to coordinate adaptive immune responses. While potential TNAs in the translational pipeline may be impeded by the prominence of these nucleic acid receptors, they also have the opportunity to utilize these natural pathways for the favorable activation of cytokine and interferon production. Favorable immunostimulation can be directed to invoke responses for use in immunotherapy, vaccine adjuvants, and antigen delivery[13].

Pattern Recognition Receptor Ligand Characteristics.

TNAs offer a unique platform for the strategic design of immunomodulatory formulations based on the known characteristics of PRR ligands. PRRs discerningly identify nucleic acid ligands due to subcellular localization, composition, nucleoside characteristics, structure, length, and sequence motifs (Table 1). Although PRRs primarily recognize nucleic acids through interaction with their sugar-phosphate backbone in a sequence-independent manner, these sensors are still able to distinguish some characteristic features of nucleic acid ligands.

RNA ligands are recognized by the sensors TLR3, TLR7, TLR8, MDA-5, and RIG-I. Within the endosome, the recognition of RNA ligands is initiated by their binding to TLRs 3, 7, or 8 which occurs at relatively low pH (<6.5). The activation of TLR3 requires interactions with dsRNA of at least 45 bp long [17] while TLRs 7 and 8 display their preferential recognition for GU and AU-rich ssRNA sequences with a minimal length of 19 bp[18–21]. There is also evidence which suggests that TLR7 can recognize short dsRNAs[22]. In addition to a binding site for RNA, TLRs 7 and 8 uniquely possess binding sites for guanosine and uridine[19, 23–26] and as such, can be activated by small synthetic molecules[27]. In the cytosol, the RLRs MAVS, MDA-5, and RIG-I recognize dsRNA ligands. Despite the fact that both MDA-5 and RIG-I possess two CARD domains, a helicase domain, and a regulatory domain, these two sensors identify distinct RNA ligands[28–30]. RIG-I binds dsRNAs longer than 19 bp through the interaction between the helicase and regulatory domains with the RNA backbone. Additionally, a 5’ di- or triphosphorylation of RNA is required for binding to the regulatory domain of PRRs[29, 31–34]. Similarly, MDA-5 recognizes RNA in a sequence-independent manner; however, it requires long dsRNAs (>1000 bp) for MDA-5 dimers to form filaments[34–36].

DNA ligands are recognized in the endosome by TLR9 and in the cytosol by several DNA sensors including cGAS, IFI16, and AIM2. TLR9 binds DNAs greater than 15 bp in length with unmethylated CpG dinucleotides that are infrequently found in mammalian DNA, but prominent in pathogen DNA[37]. Notably, naturally occurring phosphodiester

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oligodeoxynucleotides can activate TLR9 via the 2'-deoxyribose backbone independent of CpG motifs, suggesting that subcellular localization of TLR9 to the endosome is essential for discrimination of self and non-self-nucleic acids[37–39]. Additionally, TLR9 activation is enhanced in response to nucleic acid-histone complexes and supercoiled plasmids, indicating preferential binding to curved DNA ligands[37]. Finally, TLR9 is activated in response to DNA-RNA hybrids that are GU-rich and contain CpG motifs[40].

In the cytosol, cGAS binds B-form dsDNA with a minimum of 25 bp in length for efficient activation[41–43]. Recognition of dsDNA is sequence-independent, as electrostatic interactions with the sugar-phosphate backbone facilitate the interaction with two cGAS binding sites[41, 42]. Notably, cGAS can also recognize DNA/RNA hybrids and Y-form DNA, a ssDNA stem loop structure containing guanosines[44, 45]. Similar to cGAS, IFI16 and AIM bind dsDNA in a sequence-independent manner[46–50]. Both of these sensors oligomerize and form filaments along dsDNA which requires a minimum of 70–80 bp. However, optimum activation requires 150–200 and 280 bp for IFI16 and AIM, respectively[47–49, 51]. RNA polymerase III is unique in that it activates an immune response by transcribing AT-rich dsDNA into a ligand for the RNA sensor RIG-I. Importantly, AT-rich regions are required for promoter-independent initiation of transcription to generate a ligand for RIG-I[52–54].

Collectively, these PRRs form the first line of immune defense and are able to survey the cell to identify a wide range of nucleic acid ligands in order to coordinate innate and adaptive immune responses. Based on these known ligand requirements for PRR activation, nucleic acid nanoparticles (NANPs) can be rationally engineered with desired composition, nucleoside characteristics, structure, dimensionality, length of single- or double-stranded regions, and sequence motifs to generate a library of immunomodulatory, biocompatible agents suitable for a broad range of biomedical applications.

Nucleic Acid Nanoparticles (NANPs).

With known base pairing rules and an assortment of resolved naturally occurring motifs, nucleic acids can be designed to assemble into programmed NANPs of desired shapes, sizes, and compositions. An array of such structures have been demonstrated as biocompatible materials for a number of applications, encompassing biosensing[99, 100], drug delivery[101, 102], and as molecular devices[103, 104]. Depending on the design principles and motifs in their composition, NANPs can be assembled entirely from RNA or DNA, or take advantage of hybrid RNA/DNA combinations[105]. Currently, all NANPs can be roughly categorized by two design strategies[106]. The first design strategy, represented by cubes (Fig. 2A), is based solely on intermolecular canonical Watson-Crick interactions and utilizes ssRNAs and/or ssDNAs designed to assemble only with their partner strands and avoid any intramolecular secondary structures[107–109]. These design principles are widely employed in DNA nanotechnology and DNA origami[110, 111] to construct a variety of DNA NANPs. The second strategy, represented by RNA rings (Fig. 2B), is characteristic mainly of RNA nanotechnology and takes advantage of naturally occurring RNA structural and long-range interacting motifs (*e.g.*, RNAI/II inverse RNA kissing loops needed to assemble RNA rings[112]). This design strategy follows empirically rationalized rules to

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combine, similarly to Lego bricks[113–116], pre-folded RNA motifs (typically formed *via* intramolecular base-pairing of individual RNA strands) and promote their bottom-up intermolecular assembly with remarkable degree of structural control[117]. In addition to conventional nucleic acids, NANPs can also be composed of chemically modified oligonucleotides which may serve to increase their stability or be modified with fluorophores or small ligands for precise tracking and targeting[118].

When multiple TNAs are chosen for simultaneous delivery to the diseased cell (*e.g.*, for combinatorial RNAi[120]), the optimal route of their controllable formulation would be through the their attachment to the individual strands entering the composition of NANPs. Assembly of NANPs would then bring the desired TNAs together with control over their composition and stoichiometry[121], an ability confirmed by several animal trials[105, 119, 122–127]. For example, the functionalization of RNA rings with multiple siRNAs occurs through introduction of Dicer Substrate RNAs (DS RNAs)[128] designed to promote the intracellular Dicer-assisted release of experimentally validated siRNAs[119] (Fig. 2C–D). The functionalization protocols[106] include the extension of the 3'-side of individual monomers with either sense or antisense strands of DS RNA and their further annealing to the complementary strands. Following the same rationale, programmable NANPs also allow for controlled functionalization with different aptamers, fluorescent dyes, proteins, and TNAs[129–131].

Immunomodulation with NANPs.

Stemming from their nucleic acid content, NANPs with biological applications interact with PRRs in the same immune syntax as innate nucleic acids. Therefore, known patterns of NANP recognition can be used to direct their design[11, 132]. Historically, the unknown immunorecognition of nucleic acid constructs has been a major impediment to their further clinical development, with indications that NANPs may need to be classified as a new class of drugs distinct from traditional TNAs[13]. Now, pattern recognition as it relates to NANP design is being thoroughly explored to allow for more control over which interactions with PRRs can occur, which also controls their downstream signaling and cytokine production. While the design of NANPs is already imbued with the ability for innate immunorecognition, NANP scaffolds can also be decorated with known immunostimulatory oligonucleotides[133] or used to present immunogens with spatial precision[134].

Control over the various design aspects allows for NANPs to be tailored for specific immunostimulatory control. For example, one approach (Fig. 3) has been to utilize hybrid NANP fibers which reassociate in the cellular environment for the formation of double-stranded NF-κB decoy DNA duplexes while releasing multiple RNAi inducers[135]. This strategy allows for faster processing of the subsequent downregulated immunostimulation over traditional gene silencing approaches since the products require no additional processing. Various aspects of NANP design can be strategically controlled in this manner for tailored immunomodulation. Working within the cell's innate nucleic acid recognition capacity to do so allows for NANPs to serve as a molecular language for immune stimulation.

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PRRs display cell type-dependent expression and specific localization to subcellular compartments[31, 35, 43, 52, 136–139]. Cell type-dependent differences in receptor expression impact immune mediator production. Data from freshly collected human peripheral blood mononuclear cells (PBMCs) indicates NANPs are primarily identified by pDC that only express TLR7 and TLR9 in the endosome[140, 141]. Additionally, PRRs are localized to specific subcellular compartments, including the endosome and the cytosol[31, 35, 42, 43, 45, 52, 136, 137, 142, 143]. In PBMCs, delivery of NANPs via an endocytic pathway drives the production of IFNs, however, bypassing the endosome using an electroporation method of delivery abrogated any IFN responses[140]. These data suggest subcellular trafficking of NANPs may influence exposure to PRRs and thereby the production of cytokines and interferons or lack thereof. Notably, some NANPs can enter cells in the absence of a carrier, while other NANP formulations require a carrier to overcome the charge repulsion between the phosphate backbone and host cell membranes[102, 144–153]. Recent data indicates carrier-NANP combinations affect both delivery efficiency and enrichment to subcellular compartments[147]. Interestingly, NANPs composed of different nucleic acid compositions but delivered with the same carrier displayed differences in enrichment to the cytosol and endosome[147]. This provides evidence that carrier-NANP combinations are an additional parameter to direct delivery to specific subcellular compartments and either avoid or target specific PRRs.

Chemical composition and immunorecognition of NANPs.

Most PRRs display preferential recognition of RNA vs DNA due to secondary or tertiary structures and nucleoside characteristics of nucleic acids[154, 155]. Nucleic acids are composed of nucleotides that have a phosphate, a sugar (ribose or deoxyribose), and a base (adenine, guanine, cytosine, thymine, uracil). dsDNA is most commonly found in a B-form double helix. In contrast, RNA/DNA hybrids and dsRNAs exist in A-form that is highly compact due to more efficient base-pair stacking. DNA can also exist as a Z-form left-handed helix due to alternating purine-pyrimidine nucleotides $(GC)_n$ and high salt concentration[156]. Consistent with PRRs' preferential recognition of nucleic acid composition, NANP polygons that differ only in composition (RNA vs DNA vs RNA/DNA hybrid) but have the same size, shape, and connectivity induce significant differences in the production of proinflammatory cytokines and interferons[157, 158]. DNA induced minimal production of these immune modulators and immunostimulation increased with incorporation of RNA strands. Polygon NANPs composed of only RNA strands displayed the highest production of proinflammatory cytokines and interferons[157, 158]. To advance the current understanding of properties that contribute to NANP immunomodulation, quantitative structure activity relationship (QSAR) modeling was applied for NANP studies[158] (Fig. 4). Investigation of a focused panel of RNA, DNA, and RNA/DNA polygons demonstrated that NANP physicochemical properties including molecular weight, melting temperature, and half-life predict NANP-stimulated immune responses. Continued characterization of larger NANP panels can be used to expand this predictive model in order to generate a set of design parameters for engineering NANPs with desired immunostimulatory properties. Notably, in vitro and in vivo studies demonstrate polygon and three-dimensional pRNA-based NANPs stimulate minimal production of

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proinflammatory cytokines, suggesting composition is not the sole factor determining immunostimulatory properties[159–162].

Due to the sensitivity of RNA to nuclease degradation, chemically modified strands are often incorporated to increase enzymatic stability of TNAs[163–166]. Modification of the 2’-hydroxyl group with 2’-fluoro, 2’-deoxy, or 2’-O-methyl prevents nuclease recognition of RNAs[167]. Additionally, these nucleoside modifications alter PRR receptor identification and have to be considered as an additional descriptor in the NANPs’ and TNAs’ immunomodulation. The 2’-hydroxyl uridine has been demonstrated to be central to endosomal TLR recognition of RNA[167]. TLRs 3 and 7 activation in response to a 140 bp dsRNA ligand was abrogated by the incorporation of 2’-fluoro or 2’-O-methyl pyrimidines[168]. However, RIG-I activation was enhanced by these modifications. Interestingly, 2’-O-methyl modified ligands have also been demonstrated to possess TLR7 antagonistic effects which are able to reduce cytokine and interferon responses to a small molecule TLR7 agonist[169]. Consistent with these findings, 2’-fluoro-modified RNA polygons display RIG-I agonist activity and avoid activation of endosomal TLRs[147]. Additional chemical modifications can include small molecules such as cholesterol to further direct the immune responses. Cholesterol-tagged lipid-DNA nanobarrels have recently been shown to selectively bind white blood cells in the PBMC population in order to suppress the immune response upon interactions with lipopolysaccharides[170].

Architectural parameters and immunorecognition of NANPs.

Although many PRRs recognize nucleic acid ligands in a sequence-independent manner via interaction with the sugar phosphate backbone, several PRRs display preferential binding to specific sequences or sequence motifs. In the endosome, TLR7/8 display preferential recognition for GU- and AU-rich sequences while TLR9 recognizes CpG motifs with unmethylated CG dinucleotides[19–21, 26, 37, 38]. NANPs, hydrogels, polypod structures, and spherical nucleic acids have been developed to deliver agonists with these sequence characteristics to the endosome for eliciting desired TLR-dependent responses (Fig. 5A) [171–173]. In the cytosol, RNA polymerase III requires AT-rich regions for promoter-independent transcription that results in a 5’ triphosphorylated ligand for the RNA sensor, RIG-I[52, 54]. Recent evidence indicates RIG-I-dependent production of pro-inflammatory cytokines and interferons in response to RNA and DNA polygons[147]. NANPs made of in vitro transcribed RNAs strands possess a 5’ triphosphates required for binding RIG-I; therefore, it is not surprising that these NANPs directly trigger RIG-I-dependent production of interferons. In contrast, DNA NANP stimulation of RIG-I-dependent responses is indirect via the involvement of RNA polymerase III dependent mechanism (Fig. 5). Notably, incorporation of RNA strands with 2’-fluoro-modified pyrimidines, known to increase NANP chemical and thermodynamic stability, also enhanced RNA polymerase III-dependent immune responses[147].

NANP size is known to influence half-life and biodistribution in animal models as smaller nanoparticles are more readily cleared via renal clearance while larger nanoparticles accumulate in the liver and spleen[174–176]. Importantly, size is a determining factor in NANP immunostimulatory properties. As NANP size increases for a polygon shape, there

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are significantly increased levels of proinflammatory cytokines released[140, 160]. One contributing factor is delivery efficiency as larger NANPs may bind more readily in contrast to smaller analogs that can exhibit reduced uptake in the absence of a carrier due to charge repulsion with the negatively charged cell membrane[140, 141, 174]. Additionally, size is an influential factor in predicting NANP immunostimulation as PRRs display length-dependent recognition of nucleic acids[17, 20, 34, 37, 42, 48, 177]. Each of the PRRs has a minimal base-pair requirement for activation. For example, TLR3 requires a minimum of 45 bp for binding and activation[17]. Recent data supports the requirement for nucleic acid strand length as polygon NANPs consisting of dsRNA containing 22 bp per side fail to elicit TLR3 while longer dsRNAs present in nanostructures trigger TLR3 dependent responses (Fig. 5) [147, 178]. In the meantime, RNA polygons stimulate TLR7 and RIG-I-dependent immune responses, which require a minimum of 19 base-pairs for activation[147].

There are several examples of PRRs displaying shape-dependent recognition of nucleic acid ligands. First, in contrast to linearized RNA, circular RNAs (circRNAs) that are ssRNAs found in microbe genomes or produced to drive expression of proteins of interest avoid stimulation of the cellular RNA sensors, RIG-I, TLR3, and TLR7/8, providing an option for an immunoquiescent delivery platform[179]. Importantly, circRNA avoidance of RNA sensor stimulation was independent of nucleoside modifications, indicating a critical role for shape in determining interactions with these PRRs. Similarly, both TLRs 7 and 9 have been demonstrated to recognize the structural features of tRNA and curved DNA ligands, respectively [37, 180, 181]. Consistent with PRR shape-dependent recognition of nucleic acid ligands, NANP immunostimulatory properties are correlated with shape and connectivity. A direct comparison of RNA polygons (triangle, square, pentagon) of identical size demonstrates pentagons are the most potent inducers of proinflammatory cytokines compared to triangles[140]. Additionally, immunostimulation increased with RNA NANP dimensionality from linear fibers to planar rings to globular cube structures (Fig. 5B) with type I IFNs being the key biomarkers produced in response to NANP internalization by phagocytes[141, 182–184]. The same NANPs were also shown to induce type III IFNs, which have never been investigated in detail for traditional TNAs[182]. Globular RNA NANPs, such as RNA cubes, were the most immunostimulatory, when compared to their DNA analogs, inducing enhanced production of cytokines. Furthermore, RNA cubes and RNA rings but not RNA fibers elicit TLR7-dependent responses, suggesting a role for shape in determining the interaction with PRRs [140, 141]. Surprisingly, the magnitude and specificity of the immunostimulation can be additionally regulated by varying the numbers and orientations of TNAs attached to each NANP[183]. Combinations of TNAs can therefore potentially yield a second level of therapeutic activity in which the codelivery of several TNAs into one target cell is met with the synergistic effect of their scaffolding. Several different TNAs may be chosen in order to orchestrate multiple effects in the cellular environment, or the higher concentration of one type of TNA can be utilized for more efficient activity.

Conclusions.

Nucleic acid PRRs identify unique nucleic acid ligands based on cellular localization, composition, nucleoside characteristics, structure, length, and sequence motifs. These PRR

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ligand characteristics provide a set of guidelines for engineering therapeutic NANPs with defined immunostimulatory properties. Supporting evidence demonstrates that NANPs' physicochemical properties and architectural parameters can be rationally designed and in turn control immunostimulation. Additionally, QSAR modeling of focused NANP panels indicates the physicochemical properties of NANPs are strong predictors of immunostimulatory properties. Continued research efforts to characterize broader NANP panels with varied sizes, chemical compositions, dimensionalities, and structures, factors demonstrated to influence PRR-dependent responses, will further strengthen these predictive models, thereby providing a molecular language that can be used to generate a vast library of immunomodulatory NANPs. Furthermore, NANPs can be functionalized with RNAi inducers and decoy duplexes to modulate PRRs' downstream signaling, providing an additional level to control immunostimulation. As such, NANPs are a promising immunotherapeutic platform.

Currently, many small molecules, antibodies, oligonucleotides, and synthetic nucleic acids are being investigated in clinical trials as PRR agonists and antagonists. As discussed above, NANPs can be rationally designed to harness both innate and adaptive immune responses via nucleic acid PRRs. PRR agonists stimulate innate cytokine and interferon responses that can shape adaptive immunity. In particular, the production of interferons promotes cellular antiviral defenses and antigen-specific adaptive immune responses to pathogens and cancer cells. Therefore, immunomodulatory NANPs could be applied as broad-spectrum antivirals, vaccine adjuvants, and cancer immunotherapeutics (Table 1). NANP activation of endosomal PRRs could also be implemented to desensitize allergic responses by directing Th1 responses in contrast to the potent Th2 and IgE-mediated inflammatory response characteristic of allergic diseases. Alternatively, in inflammatory or autoimmune diseases, NANP PRR antagonists or NANPs complexed with functional groups to diminish PRRs' downstream signaling can be utilized to control overactivation of PRR immune responses (Table 1). Additionally, these immunomodulatory NANP platforms can be accessorized with functional groups targeting disease-related pathways for a combinatorial treatment approach.

Notably, there are additional gaps in the broader adaptation of NANPs. First, in order to cross the plasma membrane for intracellular activity, many NANPs must be complexed with a carrier[141].There are many nanoparticle-based platforms for the delivery of nucleic acids that would add an additional level of tailorability depending on the resultant route of trafficking[185]. The continuous development of novel delivery vehicles has been recommended to overcome this challenge[12]. Second, NANPs must also resist nuclease degradation and traverse complex organ systems to arrive at the target site[6]. While the delivery platform may yield some bioavailability, there are also combinations of chemical modifications which can assist in this regard[147]. Chemical modifications may also serve as an important parameter to regulate NANP-PRR interactions, thereby promoting beneficial or preventing detrimental immune responses[147, 163, 186]. Finally, systemic gaps have been cited as needing to be overcome for further successful clinical translation[187]. Moving past *in vitro* models and into *in vivo* demonstrations will assist in obtaining more relevant depictions of the effectiveness of these platforms. There is also greater need for simplicity in design and manufacturing, culminating in more universal nomenclature and protocols that can be adapted. As the summation of these future directions, specific, modular, adjustable,

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reproducible, and targeted nucleic acid nanoparticles (SMART NANPs) represent the next step in nucleic acid programmability for diverse biomedical applications. The manifestation of tailorable SMART NANPs has a lot to offer for eliciting desirable immunostimulatory and therapeutic profiles.

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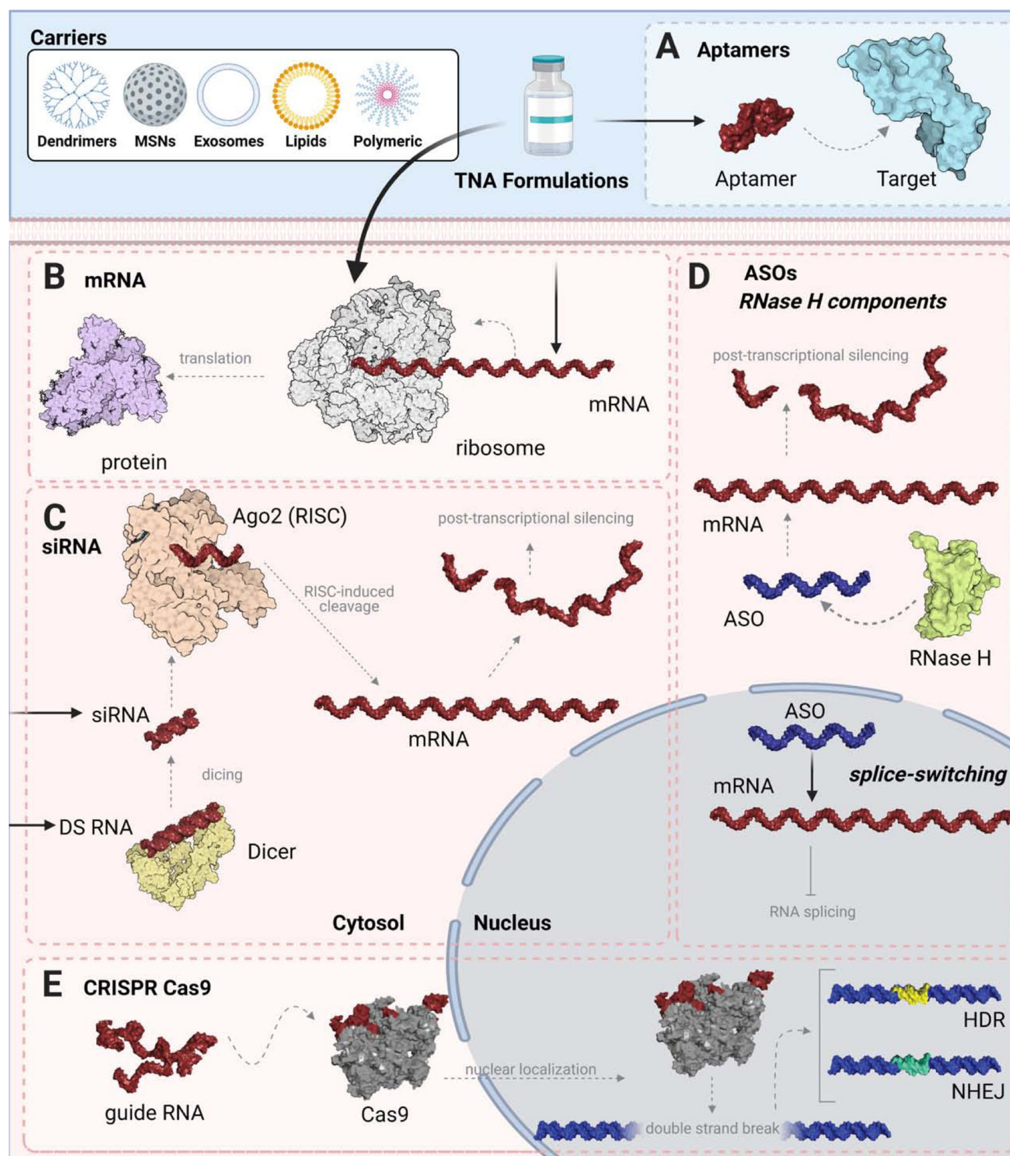

Figure 1: Examples of mechanisms of TNA action.

For efficient intracellular delivery, some TNAs require a carrier with several of them exemplified in the upper panel. (**A**) Aptamers, composed of either DNA, RNA or their chemical analogs, function by binding a specific target molecule. As an example, Pegaptanib[14] (shown using RNAComposer[15, 16]) is schematically shown to bind to VEGF (PDB: 1VPF) for its inhibition to prevent downstream angiogenesis. (**B**) Delivery of mRNA into the cytoplasm is translated via the ribosome (PDB: 6Y0G) to yield a protein of interest. Spike protein from SARS-CoV-2 (PDB: 6VXX) is shown as a protein product example of mRNA vaccines. (**C**) For RNAi-induced gene silencing, either Dicer Substrate (DS) RNAs may be introduced for processing by Dicer, or siRNAs may be introduced exogenously. siRNAs are incorporated into the RNA-induced silencing complex (RISC) and guide strands direct sequence-specific mRNA cleavage. For illustration purposes, only the Ago2 component of RISC is shown (PDB: 6CBD). (**D**) ASOs bind to an endogenous mRNA

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sequence, where they may act as a steric hinderance for further splicing and translation, or may serve as a target for degradation by RNase H (PDB: 2QK9). (**E**) CRISPR Cas9 (PDB: 5F9R) utilizes a guide RNA sequence as a template to promote the double strand breakage of a gene. Repair mechanisms including homology directed repair (HDR) or non-homologous end joining (NHEJ) can be implemented for gene editing. Created with Biorender.com

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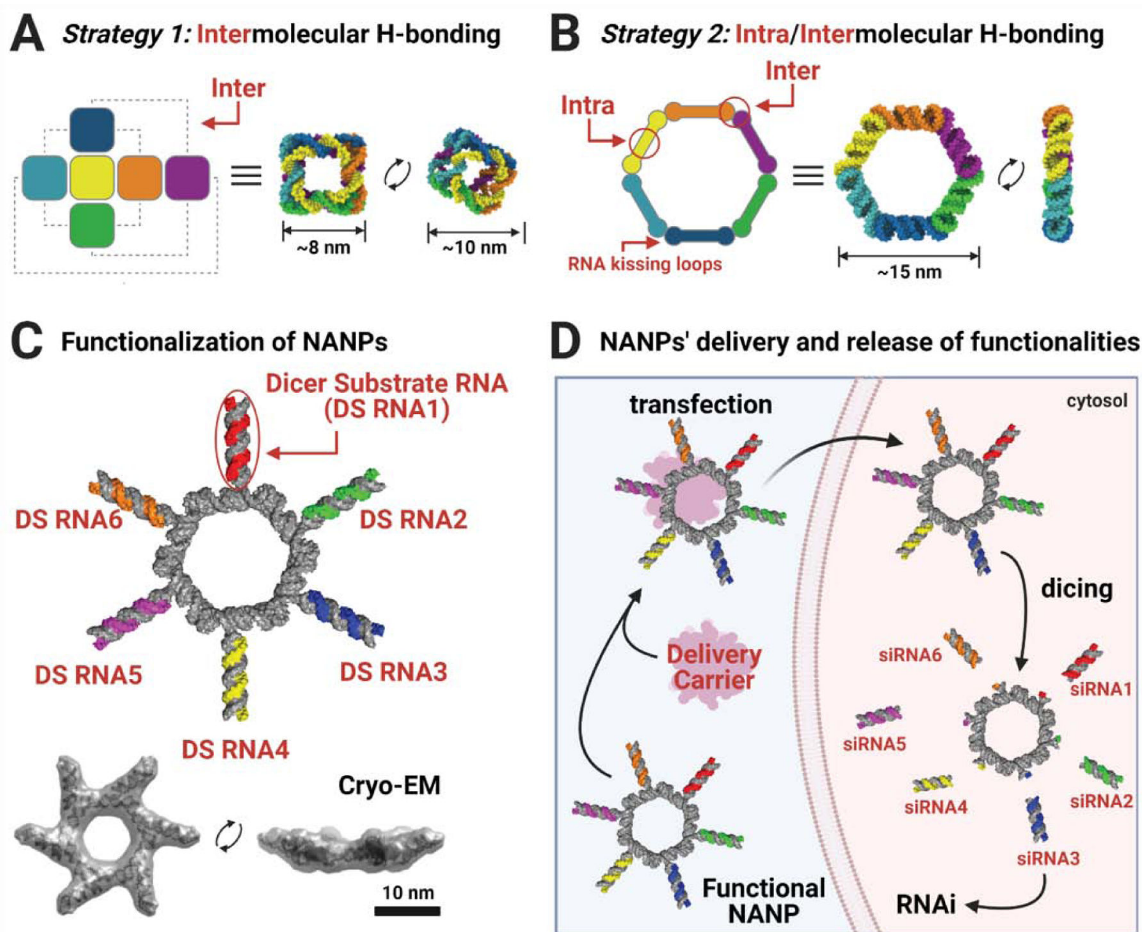

Figure 2: Design strategies and functionalization of NANPs.

(**A**) For the formation of cubes, intermolecular hydrogen bonds occur between six oligonucleotides. As these are canonical WC bps, the cubes may be composed of any combination of RNA and/or DNA. (**B**) For the formation of rings, intramolecular hydrogen bonding first occurs within each strand, exposing single-stranded regions (RNA kissing loop motifs) which can then interact intermolecularly. (**C**) By extending the sequences in their compositions, NANPs can be functionalized with Dicer Substrate (DS) RNAs which can then enter the RNA interference (RNAi) pathway. Due to their hexameric nature, up to six DS RNAs can be added to each ring for simultaneous knockdown of six different target genes. Cryo-EM (from ref. [119]) demonstrates the structure of the functional RNA rings. (**D**) Functional NANPs must be combined with a delivery carrier for their transfection into cells, where they may then be processed by Dicer to begin RNAi. Created with Biorender.com.

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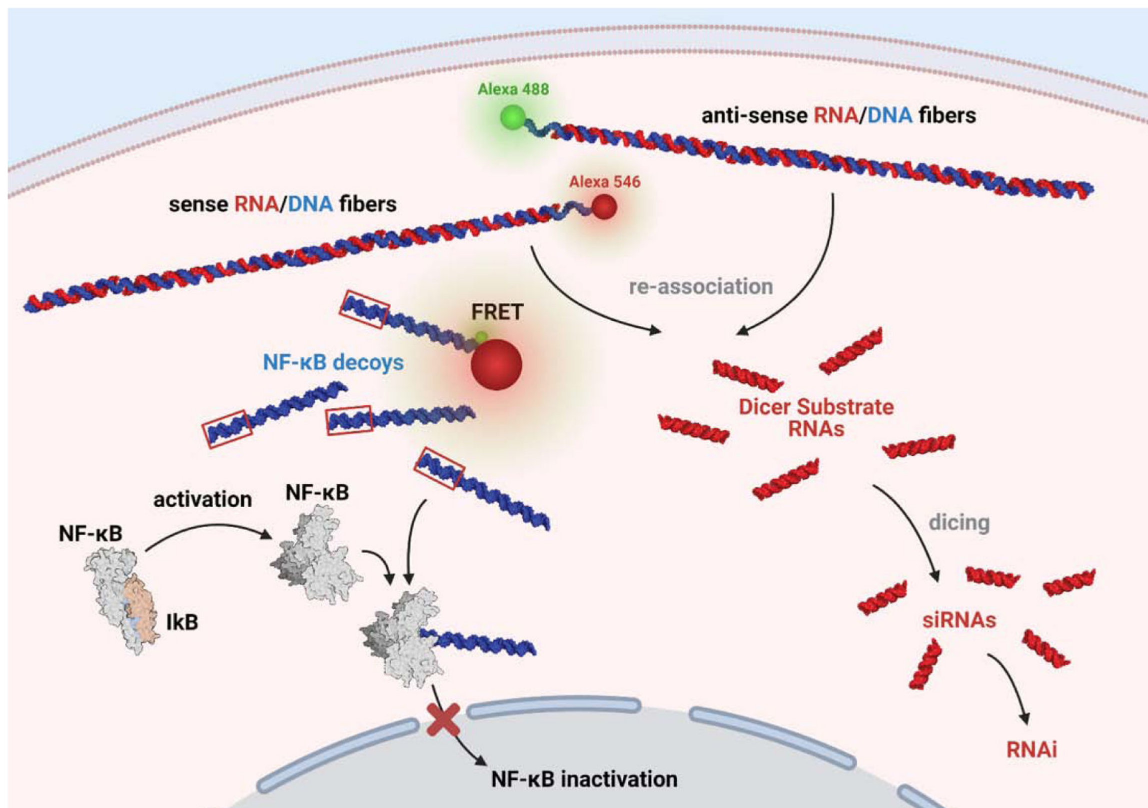

Figure 3: Non-functional RNA/DNA hybrid NANPs can be used for the coordinated activation of RNAi and NF-κB decoys.

Re-association of hybrid fibers in the cytosol yields two products. First, DS RNAs that are cleaved by Dicer produce functional siRNAs for the silencing of target genes. Second, synthetic dsDNA decoys that readily bind to NF-κB prevent nuclear translocation and activation of NF-κB-induced cytokines. PDBs: 1NFK and 1NFI. Created with Biorender.com

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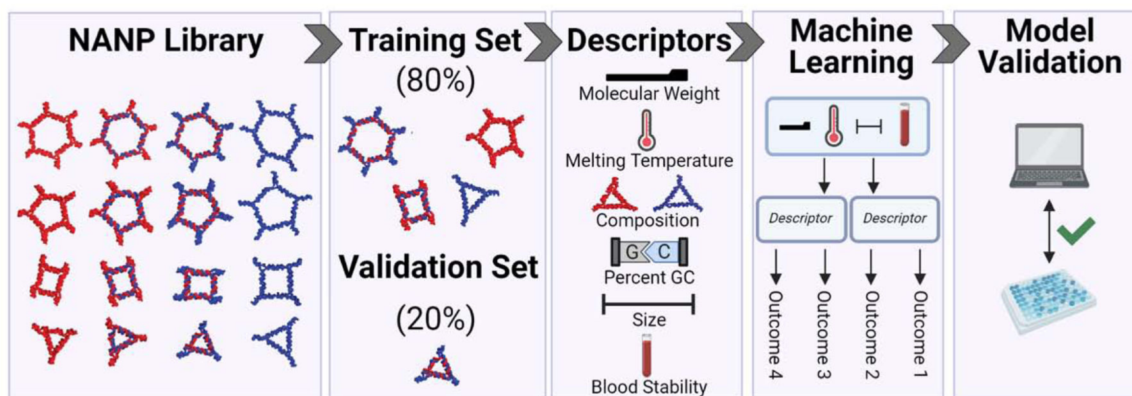

Figure 4: QSAR modeling employs a set of NANPs to predict pro-inflammatory immune responses.

A panel of representative DNA, RNA, and DNA/RNA NANPs was designed with varying descriptors such as molecular weight, melting temperature, size, and stability. A training set composed of 80% of this batch was used for machine learning, where descriptors were matched against outcomes of experimentally found immunostimulations. A validation set was then used to confirm the predicted trends and validate the model. Created with Biorender.com

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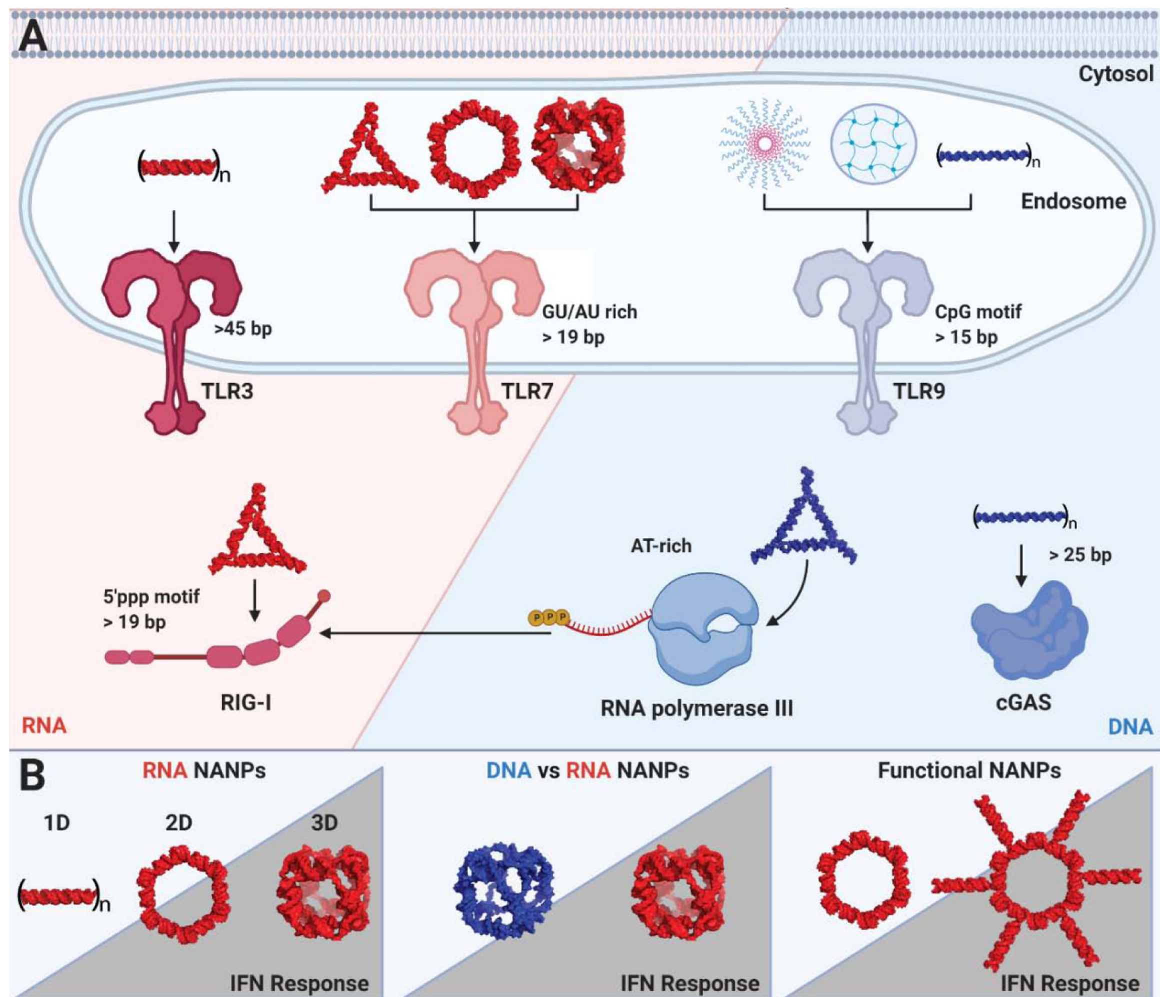

Figure 5: PRRs are localized to specific subcellular compartments to screen for PAMPs and DAMPs.

The endosomal and cytosolic sensors display composition, sequence, length, and structure-dependent recognition of nucleic acid ligands. The same ligand preferences determine binding to NANPs. The figure highlights key features of NANPs that meet the necessary ligand characteristics and have been experimentally confirmed to activate nucleic acid PRRs (**A**) with trends in relative responses in IFN productions across some representative categories of NANPs (**B**). Created with Biorender.com

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Table 1.

PRR ligand characteristics and clinical applications.

Receptor	Ligand Characteristics				Potential Therapeutic Applications	
	Localization	**Nucleic Acid**	**Sequence**	**Length (bp)**	**Agonist**	**Antagonist**
TLR3	Endosome	dsRNA	Independent	>45	**Antiviral/Adjuvant** (e.g., HIV[55, 56], influenza[57, 58], HPV[59–61]), **Cancer** (e.g., colorectal, melanoma, mammary, prostate[62, 63])	**Inflammatory disease and Autoimmunity** (e.g., SLE[63, 64])
TLR7/8	Endosome	ssRNA, dsRNA, small molecules	GU and AU rich	>19	**Antiviral/Adjuvant** (e.g., influenza, hepatitis C[27, 65, 66]), **Cancer** (e.g., melanoma, colon, lymphoma[67, 68]), **Allergy/Asthma**[27, 63]	**Inflammatory disease and Autoimmunity** (e.g., atherosclerosis, SLE ([27, 69, 70])
TLR9	Endosome	dsDNA; preference for curved DNA	Independent preference: CpG	>15	**Antiviral/Adjuvant** (e.g., malaria[71, 72], HIV[73, 74], hepatitis C[65]) **Cancer** (e.g., melanoma[75], lymphoma[76], colon[77, 78]), Asthma [63]	**Inflammatory disease and Autoimmunity** (e.g., SLE[64], HIV[79], psoriasis[80])
cGAS	Cytosol	dsDNA, DNA/RNA hybrid, Y-form	Independent	>25	STING: **Adjuvant** (e.g., influenza [81, 82], coronaviruses[83, 84]) **Cancer** (e.g., solid tumors, prostate, lymphoma [85, 86])	STING: **Inflammatory disease and Autoimmunity** ([87]
IFI16	Cytosol Nucleus	dsDNA	Independent	>70; optimal 150–200	STING: **Adjuvant** (e.g., influenza [81, 82], coronaviruses[83, 84]) **Cancer** (e.g., solid tumors, prostate, lymphoma [85, 86])	STING: **Inflammatory disease and Autoimmunity** ([87]
AIM2	Cytosol	dsDNA	Independent	>80 optimal 280	**Cancer** (e.g., colon [88, 89])	**Inflammatory disease and Autoimmunity** (e.g. psoriasis, atherosclerosis, neuroinflammation) **Cancer** (e.g., cutaneous squamous cell carcinoma, melanoma, CAR-T treatment [90]) [88, 89]
RNA pol III	Cytosol	dsDNA	AT-rich	>30		**Cancer** (colon [91])
RIG-I	Cytosol	dsRNA	5' ppp	>19	**Antiviral/Adjuvant** [92] (e.g., influenza [93], ebola[94], rabies[95]) **Cancer** (e.g., melanoma [75, 96])	**Inflammatory disease and Autoimmunity** (e.g., COPD, arthritis [97])
MDA-5	Cytosol	dsRNA	Independent	>1000	**Adjuvant Cancer** [98]	