EXPLORING THE IMMUNE SYSTEM USING HYBRID CUBE NANOPARTICLES

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ABSTRACT

JESSICA MCMILLAN. Exploring the Immune System Using Hybrid Cube Nanoparticles

(Under the direction of DR. IAN MARRIOTT)

Nano-sized cubes made from nucleic acid material (NANPs) are ideal scaffolds for therapeutic nucleic acids (TNAs). Both utilize sequence specificity through base-pairing, providing controlled strand interaction, stability, and targeted targeting delivery. However, there is a lack of research concerning nucleic acid compositions as therapeutic carrier agents and cytokine production. Exogenous NANPs and TNAs are subject to detection by immune cell receptors that can initiate undesired immune responses, threatening therapeutic reliability. The present research explores interferon-beta and interleukin-6 production induced by NANPs in human microglial cells based on nucleic acid composition and provides insight into hybrid NANPs as therapeutic drug carriers. IL-6 and IFN-β capture-specific ELISAs were used to assess immune response protein levels when cells were exposed to a 64-hybrid cube panel of 3-dimensional, six-stranded NANPs. Select hybrid NANP cubes with three RNA strands provoked significant IL-6 levels compared to carrier alone, and IFN-β production was significant for cubes NANPs with more than two RNA strands. Surprisingly, our results indicated only moderate cytokine production with increased RNA strands, an effect that was not statistically significant.

DEDICATION

To Clara Mae and Bettie.

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LIST OF ABBREVIATIONS

A

ADME

ADME absorption delivery metabolism excretion

AMP

adenosine monophosphate

ANOVA

analysis of variance

B

BBB

blood-brain barrier

C

CARD

caspase activation and recruitment domain

cGAMP

cyclic GMP-AMP

cGAS

cyclic GMP-AMP synthase

D

DNA

deoxyribonucleic acid

DS

dicer substrate

DTT

dithiolthreitol

E

EDTA

ethylenediaminetetraacetic acid

ER

endoplasmic reticulum

F

FDA

Food and drug administration

G

GFP

green fluorescent protein

H

HRP

horseradish peroxidase

I

IFN

interferon

IL

interleukin

L

L2K

lipofectamine 2000

M

MAVS

mitochondrial antiviral signaling protein

MDA

melanoma differentiation-associated protein

N

NANP

nucleid acid nanoparticle

NF

nuclear factor

P

PAGE

polyacrylamide gel electrophoresis

PCR

polymerase chain reaction

Q

QSAR

quantitative structure activity relationship

R

RIG-I

retinoic acid-inducible gene

RNA

ribonucleic acid

S

STING

stimulator of interferon genes

T

TLR

toll-like receptor

TNA

therapeutic nucleic acid

TNF

tumor necrosis factor

TRIF

TIR-domain-containing adapter-inducing interferon

CHAPTER 1. INTRODUCTION

1.1 Nucleic acids

Nucleic acids are biopolymers that play a critical role in all life forms because they possess genetic and regulatory instructions for biological pathways and gene expression. Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), composed of one (RNA) or two (DNA) polynucleotide coils made from a negatively charged phosphate backbone, a puckering ribose sugar, and four nucleotide bases, maintain and regulate the codified flow of genetic information aligned with the central dogma of biology (Figure 1.1). The dysregulation of such biological processes engenders disease states on a local and systemic level. Because RNA has emerged as a significant factor in regulating gene flow (replication, transcription, and translation), RNA-mediated therapies are now at the forefront of new therapeutic nanotechnological and biomedical applications.[1-4]

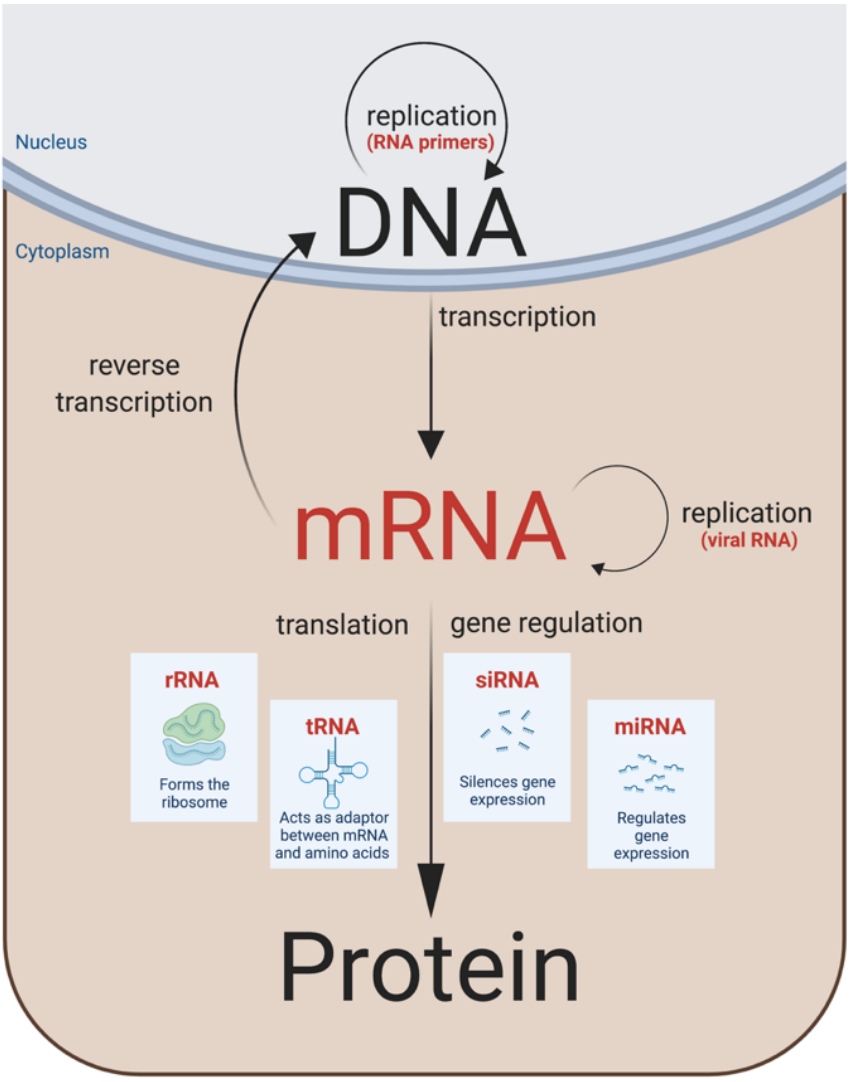

Figure 1.1 The central dogma of biology with an emphasis on RNA activity. RNA primes the replication of DNA in the nucleus. DNA is then transcribed into mRNA, and numerous RNA motifs regulate protein expression via mRNA translation.

1.2 Therapeutic nucleic acids (TNAs)

The completion of the human genome project and gene sequencing advancements makes TNAs a sensible option for directed gene therapy by thwarting protein production of problematic or targeted genes.[5] TNA oligonucleotides serving as small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), microRNAs, and aptamers, some of which are approved by the U.S. Food and Drug Administration (FDA), make up a unique group of drugs that differ in therapeutic

action due to organic chemical characteristics.[6] Aptamers designed to prevent angiogenesis (Pegaptanib) are oligomers specifically selected and enriched in vitro to recognize and bind to specific molecules as an agonist or antagonist with high affinity.[1] Additionally, there are three ASOs with RNase-H dependent binding sites and splice-switching abilities which bind to target RNAs as primers to direct strand deterioration, cleavage, or steric hindrance via RNase-H.[7, 8]

Another form of TNA is siRNA, which are short sequences that interfere with RNA translation in an enzyme-mediated fashion. There are two siRNA approved drugs, Patisiran and Givosiran. Patisiran is used to treat polyneuropathy in patients with hereditary transthyretin-mediated amyloidosis and, Givosiran addresses acute hepatic porphyria.[9, 10] More recently, mRNA TNAs, such as BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna), have been broadly distributed as vaccines for SARS-CoV-2.[11, 12]

Two organic chemistry themes govern the mechanism of action of TNA's: 1) the kinetics between the ribose pucker and negatively charged phosphate backbone, termed *dianophore,* determines the movement of drugs throughout the body and its delivery properties, or pharmacokinetics; 2) the nucleotide sequence or *pharmacophore* which determines the body's biological response and pharmacodynamics.[11, 13] Because the dianophore can undergo modifications without changing the pharmacophore's sequence in versatile oligonucleotides, pharmacokinetic properties such as absorption, distribution, metabolism, and excretion (ADME) can be altered without changing its pharmacodynamic properties; this is in contrast to traditional and information-encoded small drugs[14], in which the pharmacore and dianophore properties are dependent on one another[11, 13] restricting dynamic activity by binding pharmacokinetic properties with pharmacodynamic ones. Put more simply, if the dianophore of a small molecule drug is altered to distribute to a novel target, the body's pharmacokinetic response will also be changed

despite an unchanged pharmacophore rendering the molecular drug imprecise and unreliable. For this reason, providing independence between pharmacokinetic features (distribution and delivery) and pharmacodynamic properties (the ability to affect target cells) is a gateway to controllable and personalized medicine, starting with diversified, tissue-specific delivery of TNAs with a consistently specific function and effect.

While versatile oligonucleotides can offer more independence from base sequence and backbone modifications, they are still subject to slight alterations[15] and there is increasing proficiency in optimizing one function, such as targeted drug delivery, while preserving the subsequent pharmacodynamic effects on the target.[13]

1.3 Nucleic acid nanoparticles (NANPs)

Increased novelty and improvement of such therapeutics are significant because current clinical TNAs are costly, given in periodic doses, and promise only a single function.[11, 16] Luckily, 2D and 3D structures made from nucleic acid nanoparticles (NANPs) can be modified for simultaneous delivery.[2, 3, 17-22] Several TNA designs and assemblies have been explored wherein two- and three-dimensional scaffolds are built using nucleic acids as building blocks. The benefits of using nucleic acids extend beyond their inherent regulatory duties to their conventional and unconventional tenants in forming Watson-Crick (e.g., G—C and A—U (A—T for DNA)) base pairs because reproducible and unique NANPs[23-27] are capable of being functionalized with therapeutic cargo.[28]

Using nucleic acids as a starting material for bottom-up assembly of NANPs embraces endorses well-developed RNA nanotechnology that addresses biomedical hurdles by employing RNA for its innate biological functions and DNA for its stability.[2, 28] NANPs have shown to elicit sustained control when functionalized with therapeutic or biosensing structures (e.g.,

aptamers, proteins, and fluorescent dyes) and can deliver multiple siRNA cargoes that simultaneously target multiple pathways.[2, 25, 29] Some therapeutic NANPs have advanced to animal trials.[19, 30-32]

1.4 NANP-assisted organization of TNAs

Programmable NANPs can be designed and assembled into various shapes, sizes, and compositions using DNA, RNA, or chemical analogs, to mimic naturally occurring motifs.[30] In several studies, such biocompatible structures have been demonstrated for applications in biosensing[33, 34], molecular devices[35], and drug delivery.[36] Thanks to their engineered design, NANPs can self-assemble under biological conditions with batch-to-batch consistency, providing thermodynamic stability and delivering multiple, different TNAs simultaneously, preserving functionality.[2, 21, 28, 37-40]

TNA-NANP design promotes the specific binding of TNA oligomers to NANP monomers, preserving controlling stoichiometric control, composition, and the spatial organization of each.[41] TNA oligomers[42] bind to "sticky-ends" on the extensions of NANP monomer strands with an excess in un-paired bases that form a TNA sticky-end with specificity for an intracellular target protein. For example, in dicer substrate (DS) siRNA delivery, TNA DS antisense strands bind to NANP sense strands for simultaneous delivery. After delivery, intracellular DS antisense RNA interacts with the dicer substrate enzyme and is spliced, leading to the release of functional siRNAs. Following this approach, six DS RNAs have been documented to be successfully delivered by RNA NANP rings (Figure 1.2B) and released upon interacting with the dicer enzyme dicer[43], activating six different siRNA functions.[19]

1.5 Design principles for NANPs

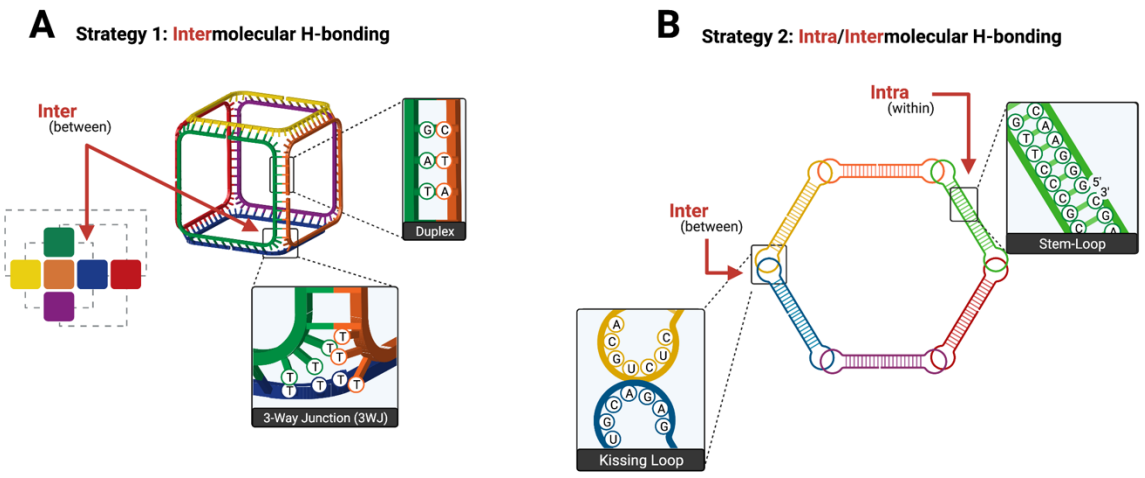

Figure 1.2 Intermolecular and intramolecular design principles for NANPs. (A) Cube NANPs have six strands that interact via intermolecular Watson-Crick base-pairing, forming a duplex around the edge sides and eight corners made with three noncomplementary thymine (DNA) or uracil (RNA) bases. (B) RNA ring NANPs are examples of both intra- and intermolecular interactions.

All NANPs can be formed using two design strategies: inter- *(between)* and intra- *(within)*, molecular interactions, and Watson-crick base pairing.[38, 44] Intermolecular hydrogen bonding (Fig. 1.2A) occurs between separate RNA and DNA strands[20, 44, 45], and are typically used in DNA origami and other nanotechnology structures.[46-48] Intramolecular interactions, exampled in RNA rings (Fig. 1.2B) and paranemic structures[49], consist of self-strand interaction, similar to long-range interacting motifs (e.g., kissing loops in RNA rings) primarily used in RNA nanotechnology.[38, 45, 50-55] Taking advantage of naturally occurring and functional RNA motifs, ring NANPs utilize both folding strategies: First, intramolecular bonding occurs with a single RNA strand, then intramolecular base-pairing between strands completes bottom-up NANP assembly.[18] Conveniently, both design strategies can utilize chemically modified oligonucleotides, fluorophores, and small ligands to improve stability or targeting and tracking, respectively.[28]

While NANPs provide cargo support and delivery, their contribution to cargo therapy-induced immune response is critical for the pharmacologic results.

1.6 Immunorecognition of NANPs

Foreign nucleic acids are considered pathogen-associated molecular patterns (PAMPs) originating from various microbes such as fungi, parasites, bacteria, and viruses. To prevent the distribution of pathogens upon infection, pattern recognition receptors (PRRs), expressed by immune cells, can recognize nucleic acid PAMPs based on nucleic acid ligand characteristics, and elicit both protective host responses and potentially detrimental inflammation.

Endosomal toll-like receptors (TLRs) can recognize many double-stranded (ds) or single-stranded (ss) nucleic acid sequences that are natural or artificial.[56] For instance, TLR7 and 8 recognize single-stranded RNA with uridine and guanine-rich regions. TLR9 recognizes unmethylated CpG DNA of more than 20 base pairs in length (Figure 1.3).[57] To prevent the detection of self-nucleic acids, endosomal TLRs follow a strict localization channel, one that bypasses the Golgi apparatus. For example, TLR-9 moves from the endoplasmic reticulum (ER) directly to an endosomal compartment after synthesis, while TLRs 3, 7, and 8 are trafficked from the ER to the Golgi and then on to lysosomal or endosomal chambers.[58, 59]

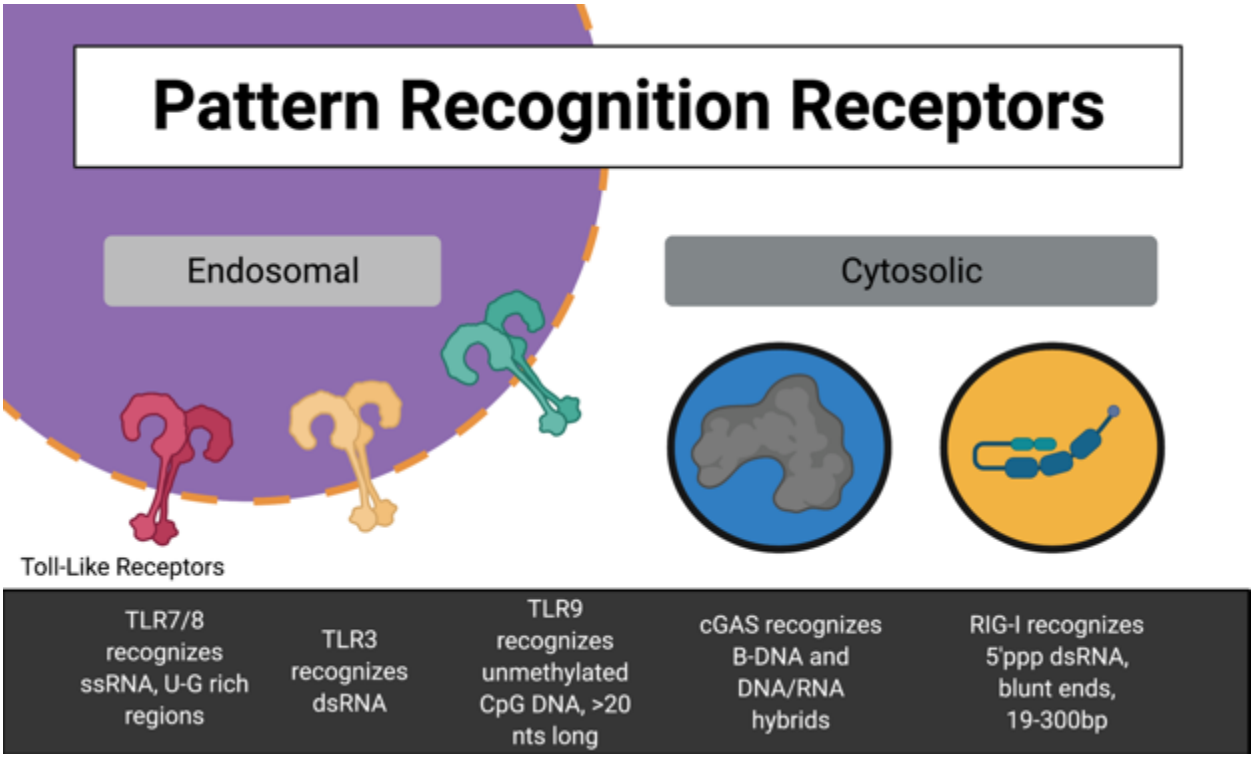

Figure 1.3 Illustration of ligand characteristics of endosomal and cytosolic Pattern Recognition Receptors (PRRs). PRRs detect nucleic acids signatures such as nucleic acid composition, strand length, and base analogs. Endosomal receptors are translocated to the lipid bilayer and are invaginated when nucleic acids are phagocytosed by cells, becoming endosomes, while cytosolic receptors are localized within the cytoplasm.

Cytosolic pattern recognition receptors like retinoic acid-inducible gene I (RIG-I) and cyclic GMP-AMP synthase (cGAS) recognize PAMPs and subsequently activate caspase activation and recruitment domains (CARD), and can recognize nucleic acids that vary in length and size, allowing for ligand-receptor specificity. For instance, the cGAS receptor recognizes dsDNA of more than 40 bp (Figure 1.3). In contrast, MDA5 (melanoma differentiation-associated protein; not pictured) recognizes long, viral dsDNA or ssRNA that are more than 1000 bp long, while RIG-I detects viral 5'tri-phosphorylated dsRNA.[60-62]

PRRs mark the beginning of a cascade of molecular interactions between receptors, adapter molecules, signaling components, and inhibitory or activating enzymes that modify the expression of immune mediators such as cytokines (Figure 1.4). For example, receptors TLRs 7,

8, and 9 interact with the adapter protein, MyD88, which creates a downstream signal to nuclear factor-kappa B (NFκ-B), inhibitor motif, liberating the p50/p60 heterodimer that translocates to the nucleus and initiates the production of inflammatory cytokines such as IL-6.[63] For cGAS, the detection of dsDNA initiates the synthesis of cyclic GMP-AMP (cGAMP), which binds to stimulator of interferon genes (STING) precipitating type I interferon expression.

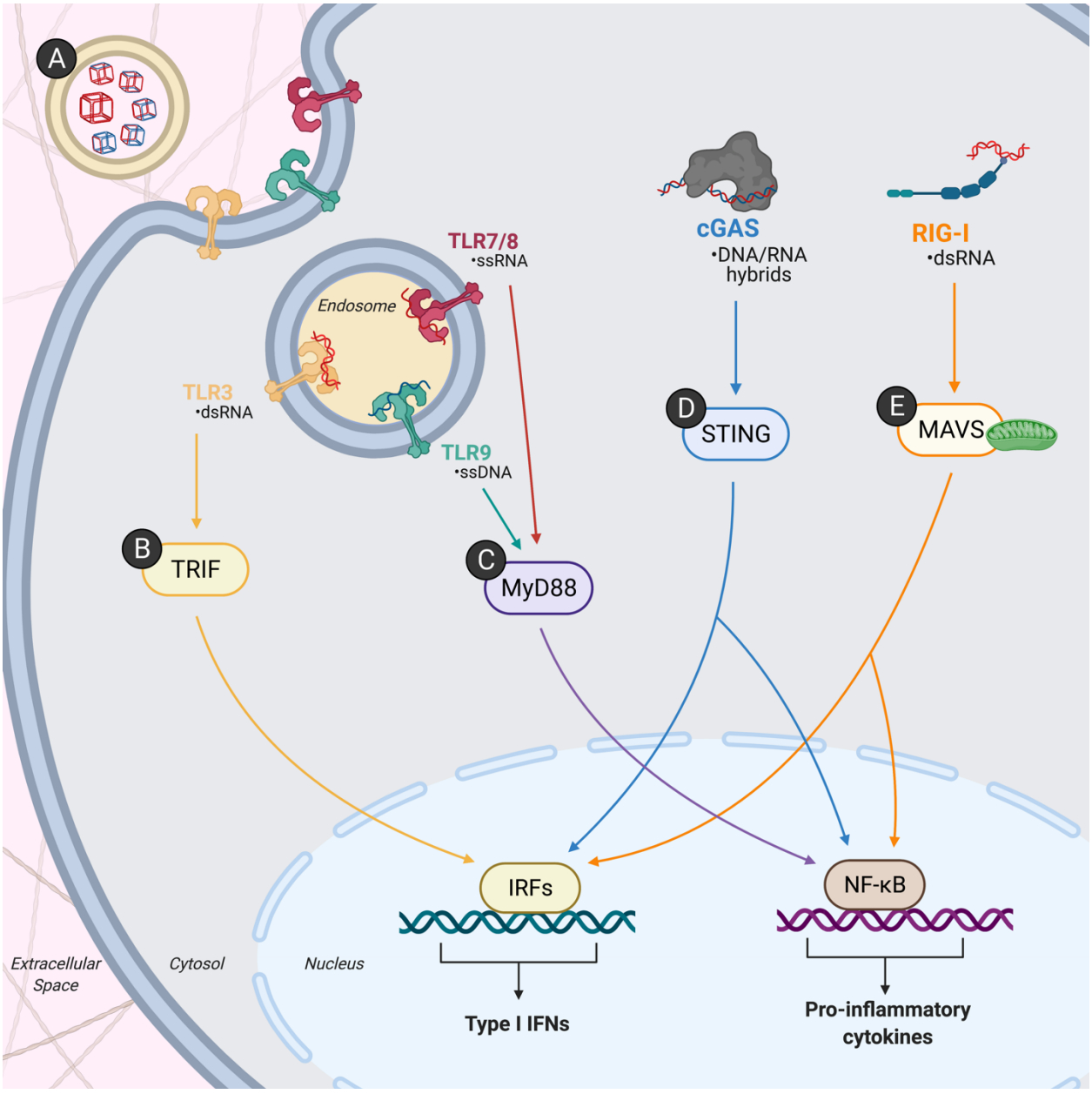

Figure 1.4 Examples of nucleic acid receptor-mediated pathways. (A) Negatively charged NANPs require a lipid carrier for entry to cells. (B) dsRNA detected by endosomal TLR 3 leads to type I IFN expression by the TRIF-mediated pathway, and (C) ssRNA and ssDNA detected by endosomal TLR7 and TLR9 are assumed to produce

pro-inflammatory cytokines via the MyD88 pathway. (D) cGAS, after detecting B-DNA and hybrid NAs, produces pro-inflammatory cytokines and type I IFNs after STING activation. (E) Pro-inflammatory NF-κB and type I IFN IRFs are expressed after RIG-I detects dsRNA.

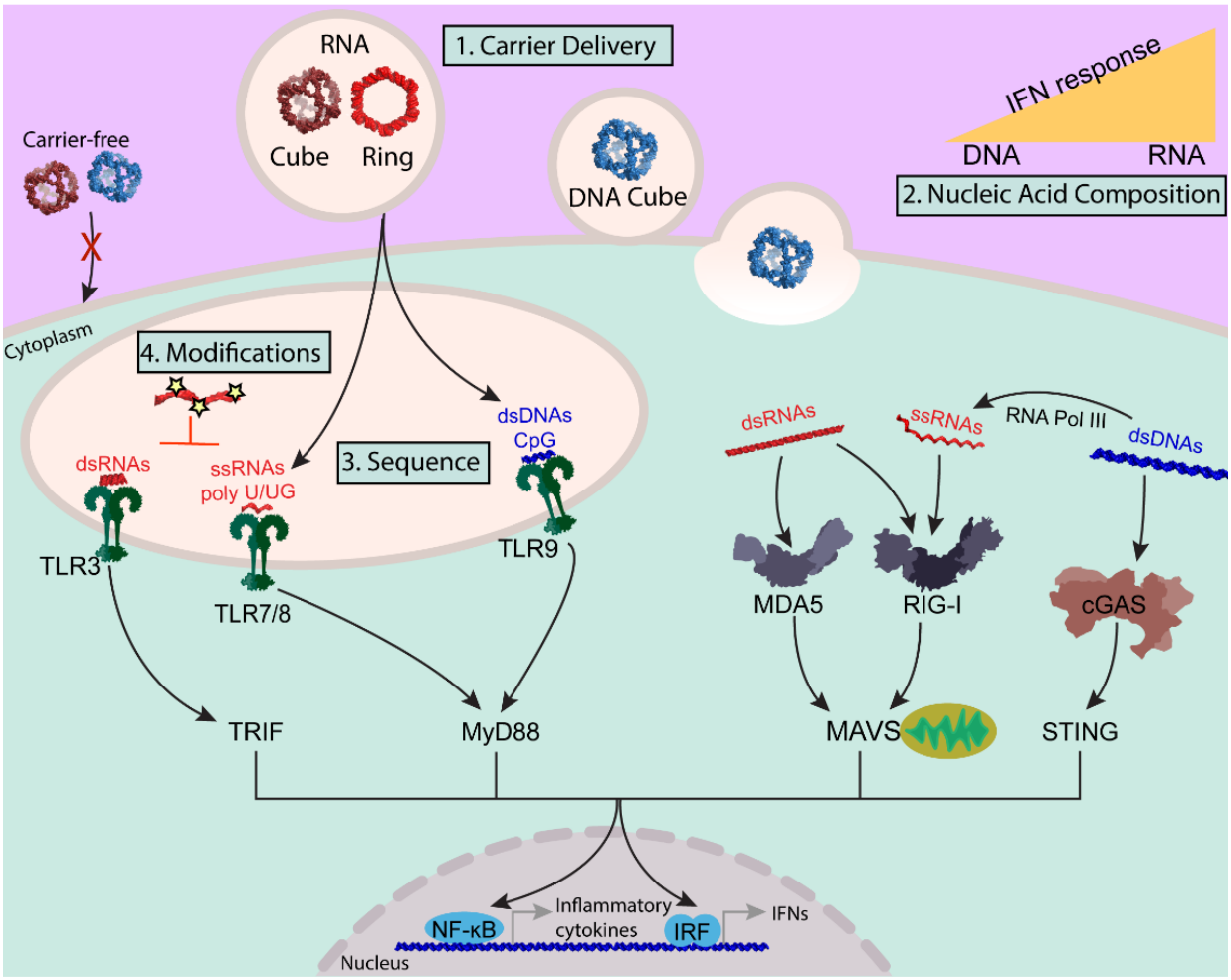

Figure 1.5 NANP carrier, nucleic acid composition, sequence, and modifications affect receptor detection. 1) NANPs must use carriers to gain entry to cells. 2) The nucleic acid composition determines the level of IFN response. 3) TLRs depend on ligand characteristics such as sequence, composition, and length of NANPs for detection. 4) Modifications to NANPs can alter the stability and TLR detection. NANPs are detected by PRRs and ultimately alter the expression of inflammatory cytokines and interferons.

RNA polymerase III transcribes AT-rich dsDNA sequences to 5'tri-phosphorylated dsRNA, which is detected by RIG-I (Figure 1.5). RIG-I is complexed with the adapter protein MAVS (mitochondrial antiviral signaling) that provides energy for the phosphorylation of IRF3 (IFN regulatory transcription factor 3) and subsequent production of type I IFNs.[64] Because the

cGAS-STING complex also activates IRF3; it too can elicit the production of type I IFNs such as IFN-β.[65, 66]

Specific interferon and interleukin production are dependent on the immune cell type as each expresses distinct PRRs. Furthermore, the activation status of such cells will also dictate PRR expression leading to variations in the threshold detection levels.[4, 67] Also, endosomal TLRs are mainly found in immune cells, while cytosolic receptors are more widely expressed and can be found in most cells. Furthermore, plasmacytoid dendritic cells (PDCs) and B-cells express higher levels of TLR7, while other immune cells such as monocytes, macrophages, and myeloid dendritic cells, minimally express TLR7 but have higher levels of TLR8.[4] This is underscored by the recent demonstration that PDCs produce the highest levels of IFN production amongst all peripheral blood mononuclear cells (PBMCs) in responses to NANPs.[56]

Furthermore, endosomal and cytosolic receptors appear to have different detection thresholds[68] in that endosomal receptors can detect lower ligand concentrations than cytosolic receptors. This is mainly due to the three-fold difference in size between endosomal and cytosolic spaces. Hence, the target location area directly affects the concentration and should be considered when determining pharmacodynamic and pharmacokinetic properties. Ligands, such as NANPs, can elicit immune responses based on the abundance of expressed receptors and their ability to recognize NANPs. Immune receptors are responsible for creating a cascade of events that directs the production of pro-inflammatory or modulatory cytokines. Cytokines, which encompass pro-inflammatory mediators and IFNs, are expressed by immune cells and can initiate or modulate protective or potentially damaging host responses and are discussed in the following sections.

Type I interferons (IFNs) are polypeptides expressed by pathogen-infected cells and carry out three main functions: (i) initiate inter-cell antimicrobial states in compromised cells and in neighboring cells, which prevent the infection of surrounding cells; (ii) alter innate immune responses in a modulatory way that encourages the presentation of antigens and natural killer cell functions but simultaneously hinders pro-inflammatory cytokine expression; (iii) commence the production of antigen-specific B and T cells by activating the adaptive immune system.[69]

For many innate immune system cells, the primary type I IFN pathway is activated by cytosolic receptors that recognize double-stranded RNA, such as RIG-I and MDA5.[70] As an example, RIG-I induced IFNs can act in an autocrine or paracrine manner to stimulate the expression of IFN stimulating genes (ISGs), which upregulate the expression of PRRs in defense against pathogens.[71] IFN-β expression is regulated by a single gene which simplifies its activation and makes it a reliable candidate for combinatorial studies as they are extensively expressed and well characterized.[72, 73]

Cytokines are also potent regulators of inflammation. Such pro-inflammatory cytokines include IL-1beta, IL-6, and TNF and are mainly produced by activated myeloid cells such as macrophages. Such pro-inflammatory chemical signals are secreted by these leukocytes and stimulate surrounding cells.[74]

During inflammation, interleukins modulate three essential functions: growth, differentiation, and activation in target cells.[75, 76] For example, IL-6 is involved in the activation of brain cells such as astrocytic and microglia.[77] Furthermore, both of these resident brain cell types can produce IL-6 following bacterial infection with *N. meningitidis* or *S. aureus*, or intracellular challenge with 5'tri-phosphorylated ssRNA.[64, 78, 79] Many of the following experiments in the present study feature the analysis of pro-inflammatory cytokine and IFN expression to determine

the relationship between NANP design and immunostimulatory activity as essential criteria to advance the clinical use of TNA-NANPs for diverse and programmable drug delivery.

In short, when triggered by ligands such as nucleic acids, receptor-mediated pathways lead to the expression of pro-inflammatory cytokines and IFN genes, modulated by ligand-specific characteristics including length, composition, modifications, and carriers. While the exploration of NANPs has shown promise for programmable therapeutic control, there are still hurdles to overcome before their use clinically.

1.7 Hurdles to the therapeutic use of NANPs

Hurdles to the clinical use of NANPs include maintaining NANP stability versus nuclease degradation, their delivery via carrier molecules, and off-target immunorecognition that can occur once therapeutic functional groups dissociate, but such roadblocks could not be overcome by determining the relationship of their structure with the induction of cellular responses.

The stability of TNAs is critical for their delivery and medicinal use. TNAs must maintain their integrity when complexed with carriers and while they traverse the bloodstream before arriving at target tissues. However, blood serum contains many enzymes that degrade foreign material such as nucleases[80], which immediately jeopardize functional biomolecules. A prior study has shown that DNA in the absence of protein and is less stable and is degraded faster than DNA complexed with protein in blood serum and saliva, suggesting NANP-protein complexes may provide serum longevity. [81] Thus, efforts to hybridize RNA using complementary biomolecules to increase TNA stability are warranted. Studies involving aptamers and split functionality hybrids have been discussed[19, 30, 35], and some have explored RNA alone for its stability and capacity to activate PRRs using an increased number of strands, strands of various lengths, and 2'-OH modifications. [45, 82-84]

Because RNAi activity does not require RNA oligomers to possess the conventional 2'-hydroxy group[85], NANP modifications to specific nucleotide regions have shown increased thermal stability along with blood serum stability[3, 82, 84, 86] and modified levels of immunogenicity.[57, 87-90] In one study, it was found that chemical modifications to the ribose or nucleobase residues such as substituting 2'deoxyuridine or thymidine rather than uridine decreased type I interferon expression in human PBMCs.[86] In addition, dendritic cells (DCs) exposed to modified nucleosides on RNA exhibited a much lower cytokine response than those challenged with unmodified RNA.[57]

Apart from chemical stability, delivery mechanisms are often unpredictable and can cause cytotoxicity or off-target effects. Some researchers have found success with immune-silent delivery by modifying RNA[91], while others have manipulated the type of cationic or lipid-based carrier used, such as dendrimers, amphiphilic co-polymers, and exosomes.[92-96]
Bulky and negatively charged NANPs must be delivered to cells using liposomes, dendrimers, exosomes, or inorganic carriers, which can also affect cell immune responses.[21, 92, 93, 97, 98] For instance, NANPs complexed with cationic dendrimers elicit the expression of danger signaling cytokines (e.g., IL-1α) rather than those associated with stress and trauma (IL-1β, IL-6, and TNF) and DNA NANPs delivered using dendrimers have a less potent immune response via stress-related cytokines than did RNA cubes.[92]

Boloamphiphiles, enhanced with stabilizing cholesterol constituents, increase the binding affinity, stability, and protection of siRNA cargo from nuclease activity, along with increased transfection efficiency and delivery across the blood-brain barrier (BBB).[94]

In exosomes, NANPs maintained their integrity for at least 60 minutes before degradation, and the delivery of NANPs was confirmed via fluorescent internalization into

human breast cancer (MDA-MB-231) cells. Furthermore, RNA NANPs designed to target GFP and complexed with exosomes elicited a marked decrease in GFP expression in MDA-MB-231-GFP cells supporting the conservation of functionality when exosomes were used as a carrier.[96] Surprisingly, the use of exosomes to deliver RNA cubes, which are known to possess potent immunostimulatory activity, failed to elicit an immune response. This makes exosomes a primary candidate for "stealth-like" delivery of therapeutic NANPs, but a less appropriate carrier for uses where immune response modulation is required. Lipofectamine, a liposomal delivery reagent, is widely used for nucleic acid transfection and delivery into many cell types for protein expression induction, functional modification, or gene silencing, with minimal cytotoxicity.[99-102] As such, these results using lipofectamine bode well for its use in limiting off-target immune system activation, enabling sustained NANP functionality in cells, and the induction of ligand-specific receptor-mediated immune responses.

NANP size, shape, and surface characteristics enable many therapeutic applications.[103] Globular shapes are responsible for cell immune stimulation compared to planar and fiber NANPs.[104]

While NANP delivery alone is generally immunoquiescent, synergistic immune-stimulation can occur when NANP-TNA complexes target receptor-mediated pathways in immune cells, and some therapeutics could trigger a robust immune response that is detrimental to the host. Clinical symptoms such as tachypnea, tachycardia, hypotension, fever, and liver or spleen distension, to name a few, are common in deregulated inflammation due to excessive cytokine expression.[105-108]

For NANP-TNA advancement, it is crucial to explore how composition, orientation, shape, and size affect immune cell recognition to understand the specific receptor-mediated pathways that prime immune stimulation and inflammation.

1.8 Quantitative Structure-Analysis Relationship (QSAR) studies of NANP immunorecognition

Cytokine production is dependent on overall NANP structures, including shape, orientation, and composition. Of these, composition is the most relevant to the present work. Specifically, DNA/RNA hybrid NANPs are of great interest due to the preservation of functional RNA and the chemical stability of DNA. To better understand this balance, immunoquiescent DNA and immunostimulatory RNA NANPs were studied by their composition to define the modulatory ranges of DNA and RNA cube immunorecognition.

Previous compositional studies focused on 2D polymers using DNA for the center and surrounding strands (D), two hybrid polygons (RcD or DcR), or RNA for the center strand and surrounding strands (R). The results report a 10- to 40-fold upregulation for IFN-β expression from polygons assembled with RNA compared to lipofectamine alone.

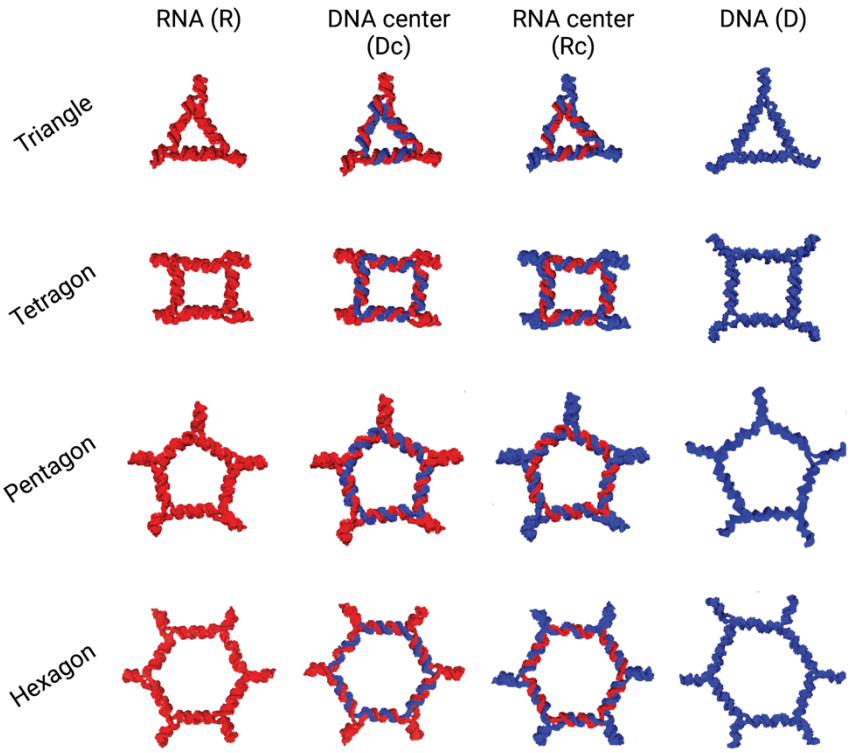

Figure 1.6 Hybrid polygon panel from quantitative structure-activity relationship (QSAR) studies. The polygon panel has 16 combinations, including four-strand triangles, five-strand tetragons, six-strand pentagons, and seven-strand hexagons. Eight polygons have hybrid compositions.

Human PBMCs with silenced TLR7 expression significantly decreased IFN response when transfected with RNA cubes and RNA rings, but not RNA fibers and DNA cubes.[56] These, and other studies, support the notion that RNA composition leads to higher immune responses to NANPs than those composed of DNA.[2, 11, 21, 28, 37, 40, 56, 109-112]

Additionally, preliminary studies in PBMCs support the idea that NANP composition determines cytokine responses to a partial panel of hybrid DNA/RNA cubes. These cubes contain five or six RNA strands, and are regarded as immunostimulatory compared to other NANPs based on type I IFN and inflammatory cytokine responses. Still, their activity does not rise to the level of eliciting a potentially fatal "cytokine storm."[113] Interestingly, IL-8 and macrophage inflammatory protein (MIP)-1α cytokine levels did not increase with additional

RNA components. Together, this data illustrates the degree to which NANP immune-stimulating properties can be controlled by mere strand composition. However, the current data do not address the full scope of composition-dependent immunostimulatory activity that could be achieved by the extensive library of hybrid nanocubes. Because of these apparent differences in cytokine production based on NANP composition, exploring all possible immune system outcomes between the two nucleic acid forms is essential.

This modular flexibility will manifest as immune regulation, thus indicating that composition directly affects cytokine production. However, the immunostimulatory activity of such NANPs must first be thoroughly characterized for us to predict the magnitude of cytokine responses for future TNA use.

CHAPTER 2. MATERIALS AND METHODS

2.1 Hybrid cube NANP design, synthesis, and self-assembly

Computer programs NUPACK and MFold were used to ensure optimal design and assembly of NANPs and predict optimal and suboptimal secondary structures of nucleic acid molecules, respectively. Computational predictions of melting temperatures (T_m) were achieved for all RNA and DNA strands using HyperFold.

All DNA starting material, including DNA cube strands, RNA cube template strands, and primers, were purchased from Integrated DNA Technologies (IDT), Inc., Coralville, IA. DNA template strands were amplified by polymerase chain reaction (PCR) using MyTaq™ mix DNA polymerase (Meridian Bioscience, Cincinnati, OH), endotoxin-free water (HyClone Laboratory, Inc., Logan, UT), and in-house buffers. Samples were annealed for 3.5 h in a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) with controlled cooling from 72 to 4°C.

Amplified DNA strands were purified using Quick DNA Kit reagents (Zymo Research, Irvine, CA) and were centrifuged for 30 seconds between washes at 10,000 rpm using an Eppendorf centrifuge 5415 D (Eppendorf, Hamburg, Germany) and evaluated using agarose gel electrophoresis dyed with ethidium bromide (EtBr) for nucleic acid visualization.

Purified DNA was transcribed with in-vitro run-off transcription (IVT) using home-made T7 RNA polymerase, rNTPs, and other necessary reagents such as 300 mM DTT, 400 mM HEPES-KOH, 10 mM spermidine, and 120 mM $MgCl_2$ at 37°C using a VWR™ Mini Block Heater (VWR International, Radnor, PA) overnight. Transcription was stopped with the addition of RQ1 RNase-free DNase (Promega Bio Sciences LLC, San Luis Obispo, CA).

Transcribed RNA and unpurified DNA strands were purified by 8M urea denaturing polyacrylamide gel electrophoresis (PAGE) using in-house ingredients. Samples were eluted in a buffer solution (300 mM NaCl and Tris-borate-EDTA) overnight at 4°C, and were added to a 1.5X volume of 100% ethanol and tempered to –20°C for 3.5 h. RNA and DNA sequences were precipitated in 2.5 volumes of 100% ethanol by centrifugation (Sorvall™ Legend™ Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA) and rinsed with 90% ethanol.

Samples were vacuum-dried using a CentriVap micro IR Vacuum Concentrator (Labconco Corporation, Kansas City, MO), dissolved in HyClone™ Water, endotoxin-free (Thermo Fisher Scientific, Waltham, MA). Cube strand absorbances were determined using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Hybrid cubes were assembled in one pot by combining equimolar concentrations (1 μM) of six DNA strands for Group 1, five DNA strands and one RNA strand for Group 2, four DNA strands and two RNA strands for Group 3, three DNA strands and three RNA strands for Group 4, two DNA strands and four RNA strands for Group 5, one DNA strand and five RNA strands for Group 6, and six RNA strands for Group 7 in 1x TB buffer (89 mM TRIS pH 8.2, 2 mM $MgCl_2$, and 50 mM KCl). Samples were annealed overnight at 37°C using a VWR™ Mini Block heater (VWR International, Radnor, PA) and snap-cooled (4°C) on ice before evaluation on 8% native PAGE at 4°C.

2.2 Cell cultures

Human microglial (hHμ) adherent cells were grown using Dulbecco's modified Eagle's medium (DMEM) (Gibco®, Waltham, MA) supplemented with 5% fetal bovine serum and 1% penicillin and streptomycin (Pen/Strep) antibiotic solution in a 5% CO_2 pressure-controlled incubator (37°C). Cells were suspended in a 5% Trypsin-EDTA (Gibco®, Waltham, MA) and

1X PBS buffer solution, plated at 5 x 10^4 cells per ml in a 12-well plate, and cultured overnight to allow cell adherence and growth.

Hybrid nanocubes were complexed with lipofectamine™ 2000 (L2K) transfection reagent in Opti-MEM™ media **(**Invitrogen™, Waltham, MA) and added to the cells. The cells were incubated at 37°C for 24 h, and the cell culture supernatant was collected and stored at -80°C for further analysis.

2.3 Cytokine secretion from hHμ C20 cells

The concentration of IL-6 and INF-β in the supernatant were revealed by enzyme-linked immunosorbent assay (ELISA) using paired reagents such as purified rat anti-human IL-6 (0.5 mg/ml) capture antibody, biotin rat anti-human IL-6 (0.5 mg/ml) detection antibody, and recombinant human IL-6 (4000 pg/ml) standard (BD Biosciences Pharmingen™, San Diego, California), and anti-interferon beta antibody (0.5 mg/ml) capture antibody, biotinylated IFN-β detection antibody (0.5 μg/ml), and recombinant human interferon-beta (2000 pg/ml) standard (Abcam®, Cambridge, UK) following the manufacturer provided protocols.

Streptavidin-HRP (horseradish peroxidase) (R&D Systems®, Minneapolis, MN), BioFX® TMB One Component HRP Microwell substrate (Avantor, Inc., Radnor, PA**)** complexes produced a colorimetric reaction that provided a graded visual stopping point at which sulfuric acid (H_2SO_4) was added. The optical density was evaluated using a Tecan Magellan™ microplate reader (Tecan US, Inc., Morrisville, NC).

2.4 Statistics

One-way ANOVA followed by Tukey's and Dunnett's multiple comparisons tests were conducted using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). A p-value of less than 0.05 was considered statistically significant.

CHAPTER 3. MANUFACTURE AND IMMUNOGENICITY CHARACTERIZATION OF A PANEL OF HYBRID NANOCUBES

As previous work[109, 110, 114] suggests, NANP characteristics such as composition, orientation, and size, contribute to the specificity and magnitude of immune cell activation. For example, the production of pro-inflammatory and immune-modulating mediators by homogenous cells correlates to nucleic acid composition as demonstrated by prior studies in which hybrid NANPs containing more RNA elicited higher levels of IFN and interleukin production than those with more DNA.[19, 110]

Because mRNA is a flexible and functional molecule carrying sequence-specific codes that regulate protein synthesis, it is a prime target for hijacking by pathogens. Thus, non-self RNA is a common ligand for pathogen motif-detecting receptors in immune cells and has the potential to serve as an immune system adjuvant, while DNA may be relatively immunoquiescent. Hybrid cubes conserve the flexibility of RNA, maintain DNA stability, and provide a platform to study immunostimulation and nucleic acid composition.

The study objectives in the present investigation of the relationship between NANP cube composition and human microglial immune cell activation were as follows:

1) To optimize a one-pot assembly method to anneal cubes with various melting temperatures.
2) Using an appropriate carrier, to transfect human microglial cells with hybrid NANP cubes and conduct specific-capture enzyme-linked immunosorbent assays to assess their immune responses.
3) To analyze and interpret the data, and to report statistically significant findings and identify trends.

A panel of 64 DNA/RNA hybrid NANP cubes (including DNA and RNA only cubes) was assembled using a one-pot method (Figure 3.1) in which cubes were assembled using six strands of 52 base-pair polynucleotides forming a "hollowed" cube-like scaffold made of DNA, RNA, or both. The cubes were organized by their DNA to RNA strand ratio: (i) six DNA strands to zero RNA strands (6D:0R) are referred to as "group 1", or "DNA cubes"; (ii) five DNA strands to one RNA strand (5D:1R) is referred to as "group 2"; (iii) four DNA strands to two RNA strands (4D:2R) are "group 3"; (iv) three DNA strands and three RNA strands (3D:3R) referred to as, "group 4"; (v) two DNA strands and four RNA strands (2D:4R) is "group 5"; (vi) one DNA strand with five RNA strands (1D:5R) is referred to as "group 6"; and (vii) zero DNA strands to six RNA strands (0D:6R) are "RNA cubes" or "group 7" (Figure 3.1).

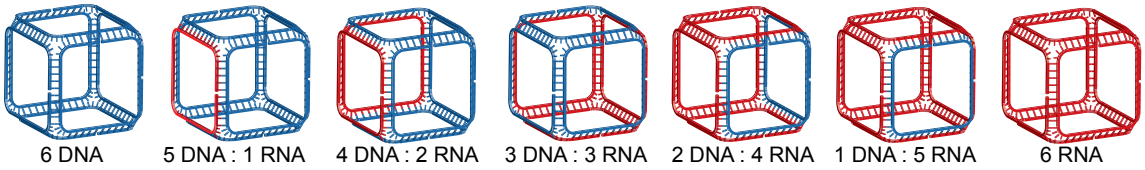

Figure 3.1 Schematic display of a truncated panel of hybrid cubes. For this project, blue indicates DNA strands, and red signifies RNA strands. From left to the right the groups and the number of cubes per group: group 1 (DNA cube, *n*=1), group 2 (*n*=6), group 3 (*n*=15), group 4 (*n*=20), group 5 (*n*=15), group 6 (*n*=6), group 7 (RNA cube, *n*=1).

Each cube was assembled using equimolar amounts of six out of twelve possible monomer strands such as "A" strand DNA named "dA-dF" for DNA strands and "rA-rF" for RNA strands; cubes were ultimately evaluated by the distance traveled, during gel electrophoresis using a non-denaturing gel, compared to cubes that were verified by electron microscopy. Figure 3.3 illustrates the verification process in which all cubes were analyzed against verified DNA and RNA cubes, left-most and right-most wells, respectively. Cubes with a single bold band in the appropriate region on the gel are considered assembled, while gel lanes with "streaks" or multiple bands indicate non-homogenous cube assemblies composed of five or fewer strands of nucleic acid (Figure 3.3).

Once verified, hybrid cubes were transfected into human microglial cells using lipofectamine as a carrier. The negatively charged phosphate backbone of the cube strands prevents passive entrance across the lipid bilayer to mammalian cells. A schematic describing the manufacture and cellular introduction of these cubes is shown in Figure 3.2.

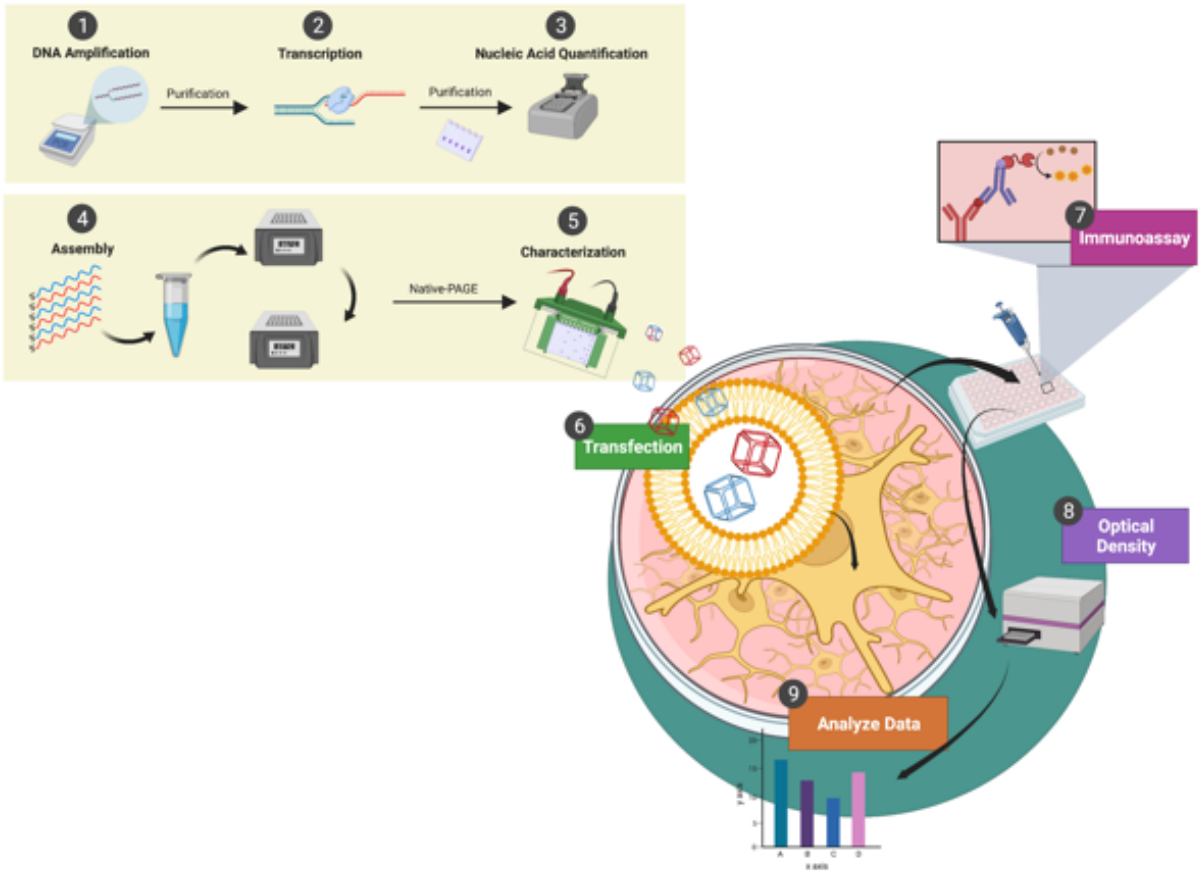

Figure 3.2 Schematic of hybrid cube manufacture and cellular introduction and evaluation. 1) DNA was amplified using polymerase chain reaction (PCR) and 2) transcribed in-vitro at 37°C. 3) Nucleic acids were quantified via spectrophotometry to ensure equimolar assembly ratios, and 4) purified strands were assembled in a one-pot method overnight (37°C). 5) NANP assemblies were verified using 8% Native PAGE and 6) transfected into cultured hHμ cells with lipofectamine (L2K) as a carrier. 7) Sample supernatant was removed in cell culture and assessed for IL-6 and IFN-β cytokine production using capture-specific ELISAs. 8) Optical density was determined using cytokine levels for respective ELISAs. 9) Data was analyzed using GraphPad Prism software.

Human microglial cells were used in these studies as they are myeloid sentinel cells of the central nervous system and express an array of PRRs that can detect single-stranded and double-stranded exogenous nucleic acids, such as NANPs, and elicit immune responses via

signaling pathways that include the MyD88 pathway.[115] Common immune system pathways lead to the production of IFN-β, a type I interferon and interleukin 6 (IL-6), a key pro-inflammatory cytokine, and hybrid cube NANPs contain many ligand characteristics for upstream PRRs that initiate the production of IFN-β and IL-6 and that are abundantly expressed in human microglial cells. After hybrid cube transfection and cell collection, hybrid cube immune responses were analyzed by IL-6 and IFN-β specific capture ELISAs.

Previous studies showed hybrid polygons (Figure 1.6) elicit more IFN than both DNA and RNA polygons; thus, it was expected that DNA/RNA hybrid cubes would have higher levels of IFN-β, compared to RNA and DNA cubes, and RNA only cubes would elicit the highest levels of IL-6 production compared to all samples.

CHAPTER 4. RESULTS AND DISCUSSION

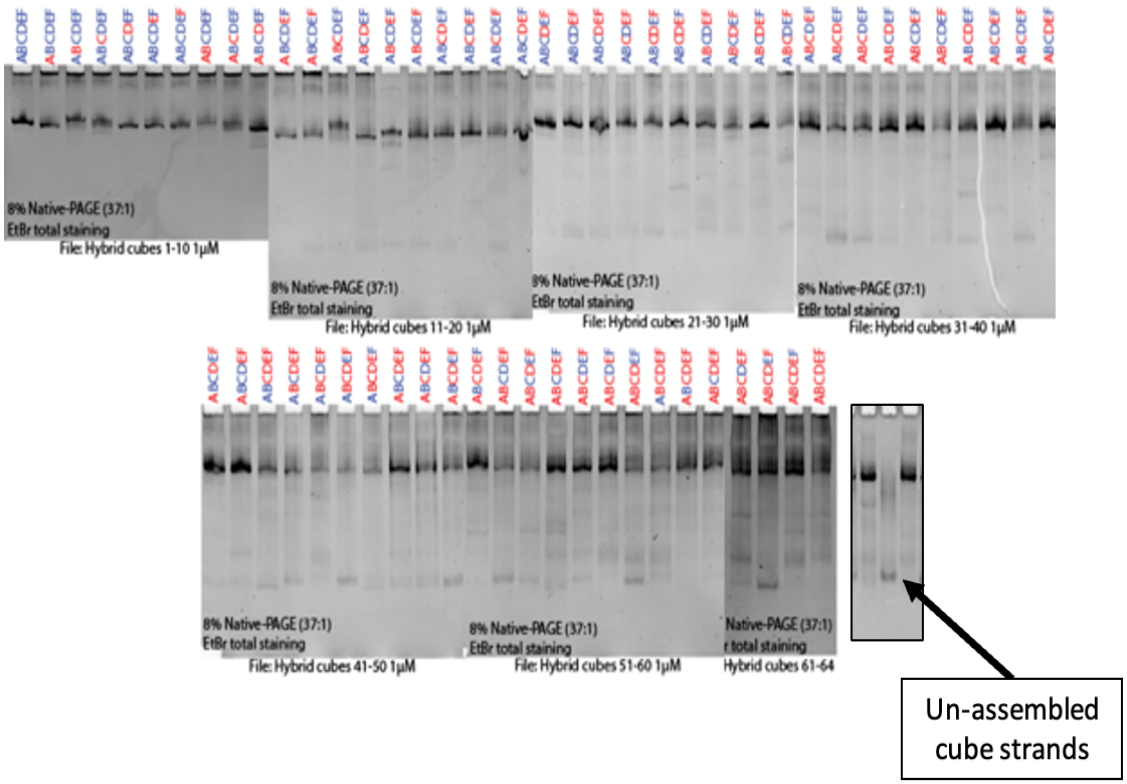

Figure 4.1 Hybrid cubes assembly verification on 8% non-denaturing gels. Ethidium Bromide staining provided a UV shadow for visualization. DNA and RNA strands "A-F" are listed in blue for DNA and red for RNA. Un-assembled cubes will have more than one band and will lack a predominant band at the appropriate distance from the wells located at the top of the gel.

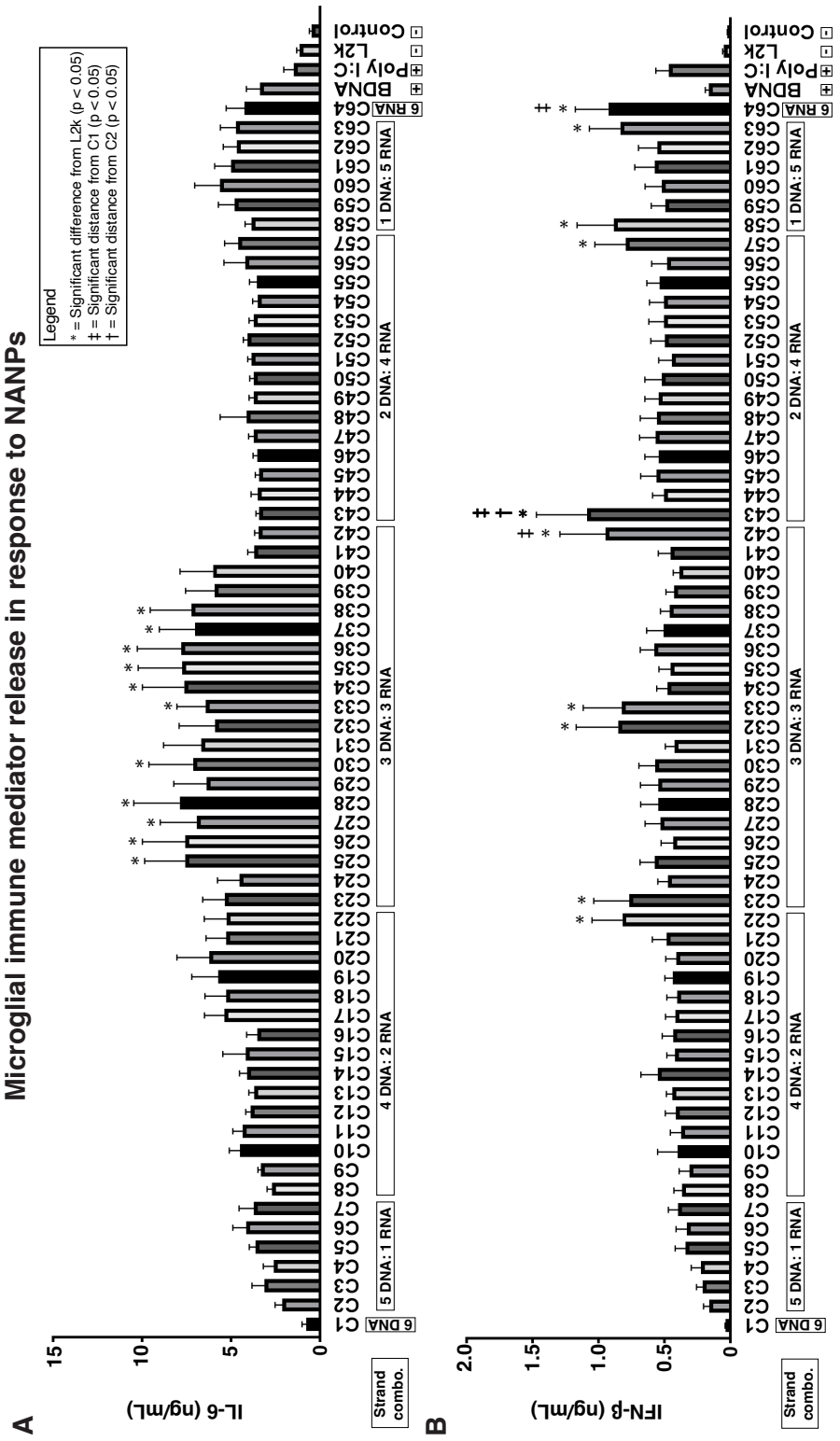

Figure 4.2 Microglial immune mediator release in response to NANPs. Human microglia cell lines were transfected with cubes using Lipofectamine 2000 (L2K) at a final concentration of 5 x 10^{-9} M. After 24-hour transfection,

supernatants were collected, and A) IL-6 and B) IFN-β induction levels from each cube C were compared against all controls and compared against each cube. Cytokine precursor expression in human microglial cells is measured in ng/ml. One way ANOVA and Dunnett tests compared cubes to "control," "L2K", and against all other cubes. One-way ANOVA and Tukey tests compared each cube to other cubes. Statistically significant results were found for select cubes compared to L2K, C1, and C2. Pictured, are the results normalized to the transfection reagent only control (L2K) presented as the mean ± SEM. Listed here and in the figure legend, there are 3 forms of statistically significant results: * = significantly different from L2K (p-value < 0.05), † = significantly different from C1 (p-value < 0.05), ‡= significantly different from C2 (p-value < 0.05).

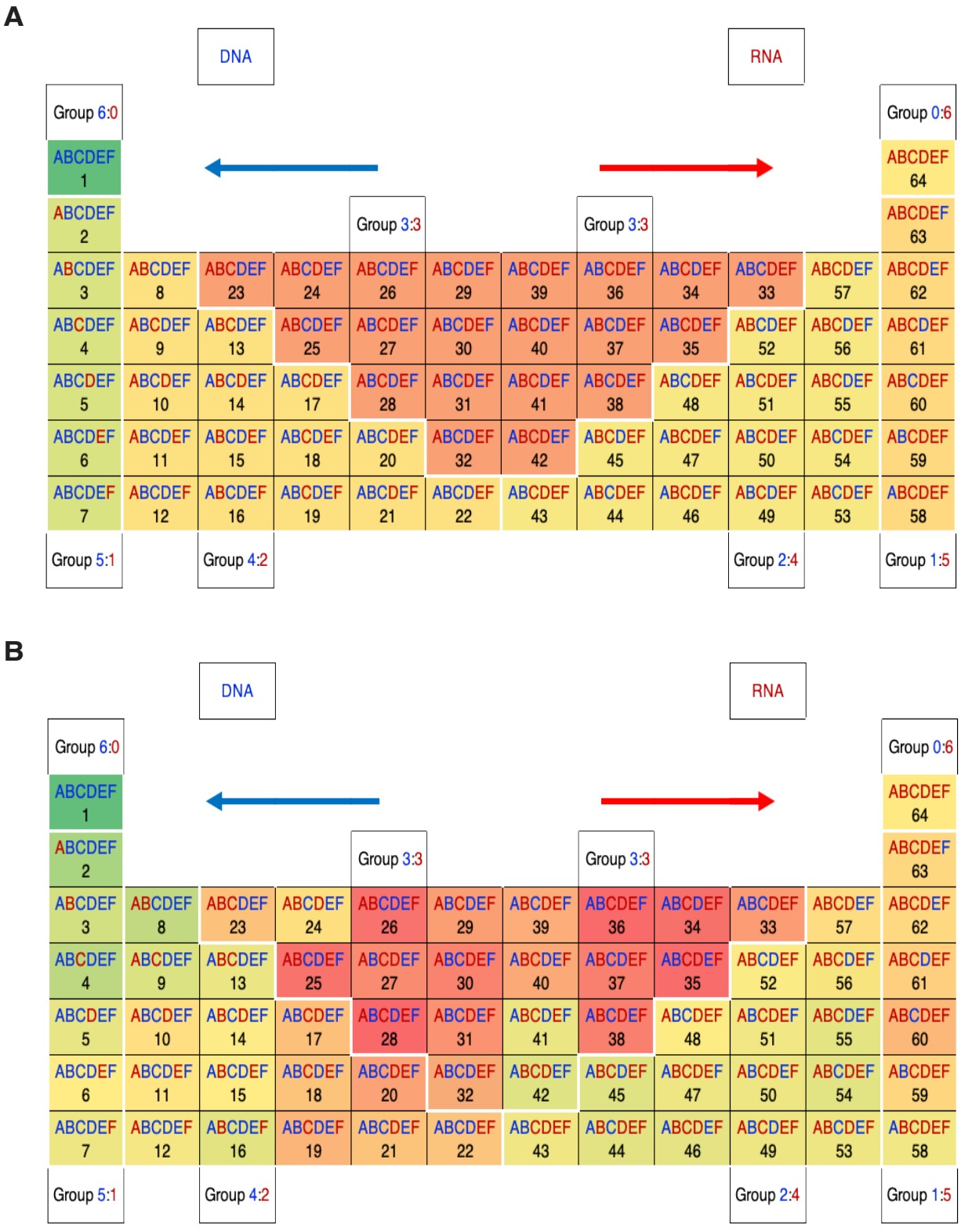

Figure 4.3 Heatmap comparison of IL-6 production between the hybrid cube panel and the L2K carrier control.

A) Average mean distance between hybrid cube-ratio groups and the L2K control. B) Average mean distance between hybrid cubes and the L2K control. Redder blocks indicate a significant difference in IL-6 production compared to the L2K control group, and greener blocks indicate IL-6 similar output compared to the L2K control group.

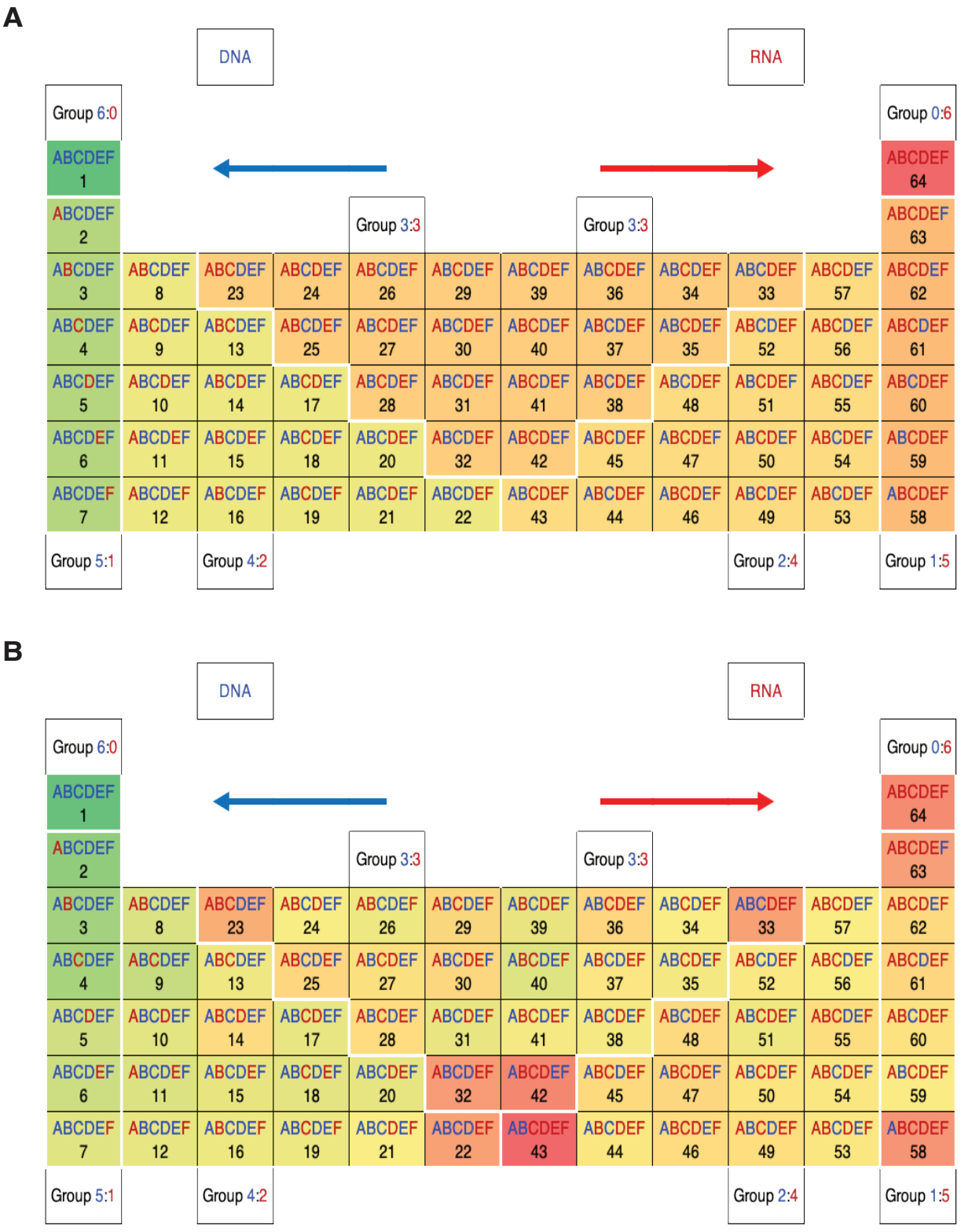

Figure 4.4 Heatmap comparison of IFN-β production between the hybrid cube panel and the L2K carrier control.

A) Average mean distance between hybrid cube-ratio groups and the L2K control. B) Average mean distance between hybrid cubes and the L2K control. The reddest blocks indicate a significant difference in IFN-β production compared to the L2K control group, and greener blocks show IFN-β similar output compared to the L2K control group.

Select hybrid cubes composed of three DNA and three RNA strands were found to produce statistically significant levels of IL-6 when compared to the L2K control (Figure 3.4). Due to the need for L2K to introduce the NANPs intracellularly, it was logical to compare the NANP-induced responses to the L2K-only treated control cells to assess their immunostimulatory activity. Hybrid cubes responsible for IL-6 production that were significantly different from cells transfected with the L2K control were only found in the three-to-three ratio group, and over 50 percent of that same group had statistically significant IL-6 levels when compared to cells transfected with L2K alone by one-way ANOVA, ($p<0.05$).

The increased levels of IFN production from cells transfected with C43, composed of two DNA strands and four RNA strands, were consistent with previous results that showed increased RNA composition positively correlates with IFN yield. However, in a multiple comparison analysis, C43 produced higher IFN levels than C64 and showed significantly more robust IFN-β production when compared to C1 and C2 ($p<0.05$). Further studies will be required to determine whether the immunomodulatory properties of C43 differ significantly from those of c64 and other cube compositions.

C42 and C43 from group 5 (2D:4R) and C64 (group 7) produced significantly higher levels of IFN-β compared to the group 1 DNA cube. The ability of these group 5 cubes and C64 (1D:5R) to produce significantly higher amounts of IFN-β was anticipated because C1 consists of only DNA strands. Additionally, when all cubes were compared to C2, C43 produced significantly higher levels of IFN-β, likely due to the increased RNA composition of C43 relative to C2, which only contains one RNA strand and five DNA strands.

For IL-6, human microglial cells transfected with group 4 hybrid cubes produced the highest levels compared to all other groups, and more than 50% of the four-stranded RNA and two-stranded DNA assemblies elicited statistically elevated levels of IL-6 when compared to L2K only treated cells as determined by Dunnett multiple comparison analysis.

Despite the expected positive correlation between RNA composition and cytokine production, hybrid cubes with three RNA strands produced the highest average of IL-6 production compared to the other NANPs, including those with more RNA strands. However, further studies will be required to determine whether RNA cubes are less immunostimulatory than hybrid cubes with fewer RNA components.

Hybrid cubes composed mainly of DNA failed to elicit statistically significant IL-6 responses compared to controls or other cubes compositions. Thus, such compositions may be well suited for use when immunoquiescence is desired.

While not statistically significant, hybrid DNA and RNA cubes with at least two RNA components averaged more IL-6 production than those composed of RNA or DNA cubes alone, and group 4 hybrid cubes with equal amounts of DNA and RNA strands jointly induced the highest IL-6 levels among the other hybrid groups. While the reason for these findings is presently unclear, it is possible that hybrid-specific nucleic acid receptors exist in microglia and that their engagement precipitates higher immune mediator production.

Moreover, reciprocal hybrid cubes (identical strand orientation, but inverse composition) C22 and C43, C23 and C42, and C32 and C33 induce statistically significant IFN-β production compared to L2K, and, despite different RNA ratios, there was no significant difference between cubes. These data suggest that DNA and RNA composition differences may be less significant than previously projected for IFN-β production.

The results from the present study will direct future studies that will expand our knowledge of the receptor-specific ligand characteristics that underlie cellular responses to NANPs and will determine the relative importance of each PRR type in the production of pro-inflammatory and immunomodulatory proteins by mammalian cells in response to these novel delivery agents.

CHAPTER 5. CONCLUSIONS

The present study found correlations between group 4 hybrid cube NANPs and IL-6 production, suggesting that hybrid cube NANPs that contain three RNA and three DNA strands elicit higher levels of pro-inflammatory molecules relative to other DNA and RNA cubes when compared to L2K controls. The cube NANPs that elicited the highest levels of IL-6 when compared to the transfection reagent alone were in the 3D:3R DNA to RNA group (Figure 3.4). A possible explanation for this finding may lie in the number of hybrid duplex edges within each hybrid ratio group. An example of this is seen in the 3D:3R hybrid cubes, in which some cubes have three DNA duplexes, three RNA duplexes, and six hybrid duplexes, or two DNA duplexes, two RNA duplexes, and eight hybrid duplexes.

Microglial possess at least two receptors for dsRNA, TLR 3 and RIG-I. Furthermore, cGAS can recognize the DNA/RNA hybrid cube sides. When one considers the PRRs that can detect foreign nucleic acids, such as these nanocubes, it is logical that those possessing more varied ligands that can be detected will elicit a greater response. For instance, the cGAS cytosolic receptor recognizes RNA and DNA duplexes. Thus, cGAS activation may elicit a different cytokine response than that induced by those that detect only DNA or RNA of a hybrid NANP. This could explain why RNA-only NANPs elicit lower responses than hybrid cubes.

In the present study, nanocubes composed of both DNA and RNA strands cause hHμ microglial cells to express higher cytokine levels than those made of DNA or RNA only, but it should be noted that, while IFN-β production appears to correlate well with RNA composition, IL-6 responses showed less sensitivity to NANP composition. It is, therefore, apparent that, while hybrid nanoparticles are a promising way to alter the expression of pro-inflammatory and

modulatory immune molecules, a significant knowledge gap remains in our understanding of the effects of DNA and RNA composition on NANP immunostimulatory activity.

The present analysis of a 64-cube hybrid NANP panel provides evidence that such NANPs can serve as ligands for multiple PRRs, with specific conformations eliciting statistically significant cytokine responses compared to controls. However, there is much more to learn about NANP characteristics that determine immune cell response, and determining the receptor-specific mechanisms that underlie their detection remains of critical importance.

REFERENCES

1. Panigaj, M.; Johnson, M. B.; Ke, W.; McMillan, J.; Goncharova, E. A.; Chandler, M.; Afonin, K. A., Aptamers as Modular Components of Therapeutic Nucleic Acid Nanotechnology. *ACS nano* **2019,** *13* (11), 12301-12321.
2. Guo, P., The emerging field of RNA nanotechnology. *Nature nanotechnology* **2010,** *5* (12), 833-42.
3. Afonin, K. A.; Kireeva, M.; Grabow, W. W.; Kashlev, M.; Jaeger, L.; Shapiro, B. A., Co-transcriptional assembly of chemically modified RNA nanoparticles functionalized with siRNAs. *Nano letters* **2012,** *12* (10), 5192-5.
4. Chandler, M.; Johnson, M. B.; Panigaj, M.; Afonin, K. A. J. C. o. i. b., Innate immune responses triggered by nucleic acids inspire the design of immunomodulatory nucleic acid nanoparticles (NANPs). **2020,** *63*, 8-15.
5. Sridharan, K.; Gogtay, N. J., Therapeutic nucleic acids: current clinical status. *British Journal of Clinical Pharmacology* **2016,** *82* (3), 659-672.
6. Roberts, T. C.; Langer, R.; Wood, M. J. A., Advances in oligonucleotide drug delivery. *Nature Reviews Drug Discovery* **2020,** *19* (10), 673-694.
7. Bennett, C. F., Therapeutic Antisense Oligonucleotides Are Coming of Age. *Annual Review of Medicine* **2019,** *70* (1), 307-321.
8. Halman, J. R.; Satterwhite, E.; Roark, B.; Chandler, M.; Viard, M.; Ivanina, A.; Bindewald, E.; Kasprzak, W. K.; Panigaj, M.; Bui, M. N. J. N. a. r., Functionally-interdependent shape-switching nanoparticles with controllable properties. **2017,** *45* (4), 2210-2220.
9. Kristen, A. V.; Ajroud-Driss, S.; Conceição, I.; Gorevic, P.; Kyriakides, T.; Obici, L., Patisiran, an RNAi therapeutic for the treatment of hereditary transthyretin-mediated amyloidosis. *Neurodegenerative Disease Management* **2019,** *9* (1), 5-23.
10. Syed, Y. Y., Givosiran: A Review in Acute Hepatic Porphyria. *Drugs* **2021,** *81* (7), 841-848.
11. Johnson, M. B.; Chandler, M.; Afonin, K. A., Nucleic acid nanoparticles (NANPs) as molecular tools to direct desirable and avoid undesirable immunological effects. *Advanced Drug Delivery Reviews* **2021,** *173*, 427-438.
12. Jackson, L. A.; Anderson, E. J.; Rouphael, N. G.; Roberts, P. C.; Makhene, M.; Coler, R. N.; McCullough, M. P.; Chappell, J. D.; Denison, M. R.; Stevens, L. J.; Pruijssers, A. J.; McDermott, A.; Flach, B.; Doria-Rose, N. A.; Corbett, K. S.; Morabito, K. M.; O'Dell, S.; Schmidt, S. D.; Swanson, P. A.; Padilla, M.; Mascola, J. R.; Neuzil, K. M.; Bennett, H.; Sun, W.; Peters, E.; Makowski, M.; Albert, J.; Cross, K.; Buchanan, W.; Pikaart-Tautges, R.; Ledgerwood, J. E.; Graham, B. S.; Beigel, J. H., An mRNA Vaccine against SARS-CoV-2 — Preliminary Report. *New England Journal of Medicine* **2020,** *383* (20), 1920-1931.
13. Khvorova, A.; Watts, J. K., The chemical evolution of oligonucleotide therapies of clinical utility. *Nature Biotechnology* **2017,** *35* (3), 238-248.
14. Cohen, J. S., Informational drugs: a new concept in pharmacology. *Antisense Res Dev* **1991,** *1* (2), 191-3.
15. Koch, T.; Shim, I.; Lindow, M.; Ørum, H.; Bohr, H. G., Quantum Mechanical Studies of DNA and LNA. *Nucleic Acid Ther* **2014,** *24* (2), 139-148.
16. Bajan, S.; Hutvagner, G., RNA-based therapeutics: from antisense oligonucleotides to miRNAs. *Cells* **2020,** *9* (1), 137.

17. Stewart, J. M.; Viard, M.; Subramanian, H. K.; Roark, B. K.; Afonin, K. A.; Franco, E., Programmable RNA microstructures for coordinated delivery of siRNAs. *Nanoscale* **2016,** *8* (40), 17542-17550.
18. Afonin, K. A.; Grabow, W. W.; Walker, F. M.; Bindewald, E.; Dobrovolskaia, M. A.; Shapiro, B. A.; Jaeger, L., Design and self-assembly of siRNA-functionalized RNA nanoparticles for use in automated nanomedicine. *Nature protocols* **2011,** *6* (12), 2022-34.
19. Afonin, K. A.; Viard, M.; Koyfman, A. Y.; Martins, A. N.; Kasprzak, W. K.; Panigaj, M.; Desai, R.; Santhanam, A.; Grabow, W. W.; Jaeger, L.; Heldman, E.; Reiser, J.; Chiu, W.; Freed, E. O.; Shapiro, B. A., Multifunctional RNA nanoparticles. *Nano letters* **2014,** *14* (10), 5662-71.
20. Afonin, K. A.; Kasprzak, W. K.; Bindewald, E.; Kireeva, M.; Viard, M.; Kashlev, M.; Shapiro, B. A., In silico design and enzymatic synthesis of functional RNA nanoparticles. *Accounts of chemical research* **2014,** *47* (6), 1731-41.
21. Parlea, L.; Puri, A.; Kasprzak, W.; Bindewald, E.; Zakrevsky, P.; Satterwhite, E.; Joseph, K.; Afonin, K. A.; Shapiro, B. A., Cellular Delivery of RNA Nanoparticles. *ACS combinatorial science* **2016,** *18* (9), 527-47.
22. Chandler, M.; Afonin, K. A., Smart-Responsive Nucleic Acid Nanoparticles (NANPs) with the Potential to Modulate Immune Behavior. *Nanomaterials (Basel, Switzerland)* **2019,** *9* (4).
23. Dibrov, S. M.; McLean, J.; Parsons, J.; Hermann, T., Self-assembling RNA square. *Proceedings of the National Academy of Sciences* **2011,** *108* (16), 6405-6408.
24. Leontis, N.; Sweeney, B.; Haque, F.; Guo, P., Conference Scene: Advances in RNA nanotechnology promise to transform medicine. *Nanomedicine : nanotechnology, biology, and medicine* **2013,** *8* (7), 1051-1054.
25. Høiberg, H. C.; Sparvath, S. M.; Andersen, V. L.; Kjems, J.; Andersen, E. S., An RNA Origami Octahedron with Intrinsic siRNAs for Potent Gene Knockdown. *Biotechnology Journal* **2019,** *14* (1), 1700634.
26. Sajja, S.; Chandler, M.; Fedorov, D.; Kasprzak, W. K.; Lushnikov, A.; Viard, M.; Shah, A.; Dang, D.; Dahl, J.; Worku, B.; Dobrovolskaia, M. A.; Krasnoslobodtsev, A.; Shapiro, B. A.; Afonin, K. A., Dynamic Behavior of RNA Nanoparticles Analyzed by AFM on a Mica/Air Interface. *Langmuir : the ACS journal of surfaces and colloids* **2018,** *34* (49), 15099-15108.
27. Geary, C.; Chworos, A.; Verzemnieks, E.; Voss, N. R.; Jaeger, L., Composing RNA Nanostructures from a Syntax of RNA Structural Modules. *Nano letters* **2017,** *17* (11), 7095-7101.
28. Jasinski, D.; Haque, F.; Binzel, D. W.; Guo, P., Advancement of the Emerging Field of RNA Nanotechnology. *ACS nano* **2017,** *11* (2), 1142-1164.
29. Shu, D.; Shu, Y.; Haque, F.; Abdelmawla, S.; Guo, P., Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics. *Nature nanotechnology* **2011,** *6* (10), 658-67.
30. Afonin, K. A.; Viard, M.; Martins, A. N.; Lockett, S. J.; Maciag, A. E.; Freed, E. O.; Heldman, E.; Jaeger, L.; Blumenthal, R.; Shapiro, B. A., Activation of different split functionalities on re-association of RNA-DNA hybrids. *Nature nanotechnology* **2013,** *8* (4), 296-304.
31. Lee, H.; Lytton-Jean, A. K.; Chen, Y.; Love, K. T.; Park, A. I.; Karagiannis, E. D.; Sehgal, A.; Querbes, W.; Zurenko, C. S.; Jayaraman, M.; Peng, C. G.; Charisse, K.;

Borodovsky, A.; Manoharan, M.; Donahoe, J. S.; Truelove, J.; Nahrendorf, M.; Langer, R.; Anderson, D. G., Molecularly self-assembled nucleic acid nanoparticles for targeted in vivo siRNA delivery. *Nature nanotechnology* **2012,** *7* (6), 389-93.
32. Guo, S.; Vieweger, M.; Zhang, K.; Yin, H.; Wang, H.; Li, X.; Li, S.; Hu, S.; Sparreboom, A.; Evers, B. M.; Dong, Y.; Chiu, W.; Guo, P., Ultra-thermostable RNA nanoparticles for solubilizing and high-yield loading of paclitaxel for breast cancer therapy. *Nature communications* **2020,** *11* (1), 972.
33. Roark, B.; Tan, J. A.; Ivanina, A.; Chandler, M.; Castaneda, J.; Kim, H. S.; Jawahar, S.; Viard, M.; Talic, S.; Wustholz, K. L.; Yingling, Y. G.; Jones, M.; Afonin, K. A., Fluorescence Blinking as an Output Signal for Biosensing. *ACS sensors* **2016,** *1* (11), 1295-1300.
34. Afonin, K. A.; Danilov, E. O.; Novikova, I. V.; Leontis, N. B., TokenRNA: a new type of sequence-specific, label-free fluorescent biosensor for folded RNA molecules. *Chembiochem : a European journal of chemical biology* **2008,** *9* (12), 1902-5.
35. Afonin, K. A.; Desai, R.; Viard, M.; Kireeva, M. L.; Bindewald, E.; Case, C. L.; Maciag, A. E.; Kasprzak, W. K.; Kim, T.; Sappe, A.; Stepler, M.; Kewalramani, V. N.; Kashlev, M.; Blumenthal, R.; Shapiro, B. A., Co-transcriptional production of RNA-DNA hybrids for simultaneous release of multiple split functionalities. *Nucleic acids research* **2014,** *42* (3), 2085-97.
36. Halman, J. R.; Kim, K. T.; Gwak, S. J.; Pace, R.; Johnson, M. B.; Chandler, M. R.; Rackley, L.; Viard, M.; Marriott, I.; Lee, J. S.; Afonin, K. A., A cationic amphiphilic co-polymer as a carrier of nucleic acid nanoparticles (Nanps) for controlled gene silencing, immunostimulation, and biodistribution. *Nanomedicine : nanotechnology, biology, and medicine* **2020,** *23*, 102094.
37. Johnson, M. B.; Halman, J. R.; Miller, D. K.; Cooper, J. S.; Emil; Marriott, I.; Afonin, K. A., The immunorecognition, subcellular compartmentalization, and physicochemical properties of nucleic acid nanoparticles can be controlled by composition modification. *Nucleic acids research* **2020,** *48* (20), 11785-11798.
38. Grabow, W. W.; Zakrevsky, P.; Afonin, K. A.; Chworos, A.; Shapiro, B. A.; Jaeger, L., Self-assembling RNA nanorings based on RNAI/II inverse kissing complexes. *Nano letters* **2011,** *11* (2), 878-87.
39. Bui, M. N.; Brittany Johnson, M.; Viard, M.; Satterwhite, E.; Martins, A. N.; Li, Z.; Marriott, I.; Afonin, K. A.; Khisamutdinov, E. F., Versatile RNA tetra-U helix linking motif as a toolkit for nucleic acid nanotechnology. *Nanomedicine : nanotechnology, biology, and medicine* **2017,** *13* (3), 1137-1146.
40. Guo, S.; Xu, C.; Yin, H.; Hill, J.; Pi, F.; Guo, P. J. W. I. R. N.; Nanobiotechnology, Tuning the size, shape and structure of RNA nanoparticles for favorable cancer targeting and immunostimulation. **2020,** *12* (1), e1582.
41. Dao, B. N.; Viard, M.; Martins, A. N.; Kasprzak, W. K.; Shapiro, B. A.; Afonin, K. A., Triggering RNAi with multifunctional RNA nanoparticles and their delivery. *DNA and RNA Nanotechnology* **2015,** *2* (1), 1-12.
42. Guo, P.; Zhang, C.; Chen, C.; Garver, K.; Trottier, M., Inter-RNA Interaction of Phage φ29 pRNA to Form a Hexameric Complex for Viral DNA Transportation. *Molecular cell* **1998,** *2* (1), 149-155.
43. Rose, S. D., Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic acids research* **2005,** *33* (13), 4140-4156.

44. Afonin, K. A.; Bindewald, E.; Yaghoubian, A. J.; Voss, N.; Jacovetty, E.; Shapiro, B. A.; Jaeger, L., In vitro assembly of cubic RNA-based scaffolds designed in silico. *Nature nanotechnology* **2010,** *5* (9), 676-82.
45. Afonin, K. A.; Kasprzak, W.; Bindewald, E.; Puppala, P. S.; Diehl, A. R.; Hall, K. T.; Kim, T. J.; Zimmermann, M. T.; Jernigan, R. L.; Jaeger, L.; Shapiro, B. A., Computational and experimental characterization of RNA cubic nanoscaffolds. *Methods (San Diego, Calif.)* **2014,** *67* (2), 256-65.
46. Seeman, N. C.; Sleiman, H. F., DNA nanotechnology. *Nature Reviews Materials* **2017,** *3* (1), 1-23.
47. Seeman, N. C., Structural DNA Nanotechnology: An Overview. Humana Press: pp 143-166.
48. Seeman, J. C. a. N., Synthesis from DNA of a molecule with the connectivity of a cube. *Nature* **1991,** *350* (6319), 631-633.
49. Afonin, K. A.; Cieply, D. J.; Leontis, N. B., Specific RNA self-assembly with minimal paranemic motifs. *Journal of the American Chemical Society* **2008,** *130* (1), 93-102.
50. Afonin, K. A.; Lin, Y. P.; Calkins, E. R.; Jaeger, L., Attenuation of loop-receptor interactions with pseudoknot formation. *Nucleic acids research* **2012,** *40* (5), 2168-80.
51. Afonin, K. A.; Lindsay, B.; Shapiro, B. A., Engineered RNA Nanodesigns for Applications in RNA Nanotechnology. *DNA and RNA Nanotechnology* **2013,** *1* (1).
52. Afonin, K. A.; Leontis, N. B., Generating new specific RNA interaction interfaces using C-loops. *Journal of the American Chemical Society* **2006,** *128* (50), 16131-7.
53. Bindewald, E.; Afonin, K.; Jaeger, L.; Shapiro, B. A., Multistrand RNA Secondary Structure Prediction and Nanostructure Design Including Pseudoknots. *ACS nano* **2011,** *5* (12), 9542-9551.
54. Bindewald, E.; Afonin, K. A.; Viard, M.; Zakrevsky, P.; Kim, T.; Shapiro, B. A., Multistrand Structure Prediction of Nucleic Acid Assemblies and Design of RNA Switches. *Nano letters* **2016,** *16* (3), 1726-35.
55. Parlea, L.; Bindewald, E.; Sharan, R.; Bartlett, N.; Moriarty, D.; Oliver, J.; Afonin, K. A.; Shapiro, B. A., Ring Catalog: A resource for designing self-assembling RNA nanostructures. *Methods (San Diego, Calif.)* **2016,** *103*, 128-37.
56. Hong, E.; Halman, J. R.; Shah, A.; Cedrone, E.; Truong, N.; Afonin, K. A.; Dobrovolskaia, M. A., Toll-Like Receptor-Mediated Recognition of Nucleic Acid Nanoparticles (NANPs) in Human Primary Blood Cells. *Molecules (Basel, Switzerland)* **2019,** *24* (6).
57. Karikó, K.; Buckstein, M.; Ni, H.; Weissman, D., Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* **2005,** *23* (2), 165-75.
58. Miyake, K.; Shibata, T.; Ohto, U.; Shimizu, T.; Saitoh, S.-I.; Fukui, R.; Murakami, Y., Mechanisms controlling nucleic acid-sensing Toll-like receptors. *International Immunology* **2018,** *30* (2), 43-51.
59. Lee, B. L.; Barton, G. M., Trafficking of endosomal Toll-like receptors. *Trends in Cell Biology* **2014,** *24* (6), 360-369.
60. Pichlmair, A.; Schulz, O.; Tan, C.-P.; Rehwinkel, J.; Kato, H.; Takeuchi, O.; Akira, S.; Way, M.; Schiavo, G.; Reis E Sousa, C., Activation of MDA5 Requires Higher-Order RNA Structures Generated during Virus Infection. *Journal of Virology* **2009,** *83* (20), 10761-10769.
61. Schlee, M.; Roth, A.; Hornung, V.; Hagmann, C. A.; Wimmenauer, V.; Barchet, W.; Coch, C.; Janke, M.; Mihailovic, A.; Wardle, G.; Juranek, S.; Kato, H.; Kawai, T.; Poeck,

H.; Fitzgerald, K. A.; Takeuchi, O.; Akira, S.; Tuschl, T.; Latz, E.; Ludwig, J.; Hartmann, G., Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* **2009,** *31* (1), 25-34.
62. Loo, Y.-M.; Fornek, J.; Crochet, N.; Bajwa, G.; Perwitasari, O.; Martinez-Sobrido, L.; Akira, S.; Gill, M. A.; GarcíA-Sastre, A.; Katze, M. G.; Gale, M., Distinct RIG-I and MDA5 Signaling by RNA Viruses in Innate Immunity. *Journal of Virology* **2008,** *82* (1), 335-345.
63. Evankovich, J.; Lear, T.; Baldwin, C.; Chen, Y.; White, V.; Villandre, J.; Londino, J.; Liu, Y.; McVerry, B.; Kitsios, G. D.; Mallampalli, R. K.; Chen, B. B., Toll-like Receptor 8 Stability Is Regulated by Ring Finger 216 in Response to Circulating MicroRNAs. *American Journal of Respiratory Cell and Molecular Biology* **2020,** *62* (2), 157-167.
64. Johnson, M. B.; Halman, J. R.; Burmeister, A. R.; Currin, S.; Khisamutdinov, E. F.; Afonin, K. A.; Marriott, I., Retinoic acid inducible gene-I mediated detection of bacterial nucleic acids in human microglial cells. *Journal of Neuroinflammation* **2020,** *17* (1).
65. Jeffries, A. M.; Marriott, I. J. N. l., Human microglia and astrocytes express cGAS-STING viral sensing components. **2017,** *658*, 53-56.
66. Jeffries, A. Role of the Novel DNS Sensors cGAS, IFI16, and ZBP1, During Viral Infections in Glia. The University of North Carolina at Charlotte, 2020.
67. Broz, P.; Monack, D. M., Newly described pattern recognition receptors team up against intracellular pathogens. *Nature Reviews Immunology* **2013,** *13* (8), 551-565.
68. Chandler, M.; Johnson, M. B.; Panigaj, M.; Afonin, K. A., Innate immune responses triggered by nucleic acids inspire the design of immunomodulatory nucleic acid nanoparticles (NANPs). *Current opinion in biotechnology* **2019,** *63*, 8-15.
69. Ivashkiv, L. B.; Donlin, L. T., Regulation of type I interferon responses. *Nature Reviews Immunology* **2014,** *14* (1), 36-49.
70. Kawai, T.; Akira, S., The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology* **2010,** *11* (5), 373-384.
71. Schneider, W. M.; Chevillotte, M. D.; Rice, C. M., Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annual Review of Immunology* **2014,** *32* (1), 513-545.
72. Trinchieri, G., Type I interferon: friend or foe? *Journal of Experimental Medicine* **2010,** *207* (10), 2053-2063.
73. Decker, T.; Müller, M.; Stockinger, S., The yin and yang of type I interferon activity in bacterial infection. *Nat Rev Immunol* **2005,** *5* (9), 675-87.
74. Zhang, J.-M.; An, J., Cytokines, Inflammation, and Pain. *International Anesthesiology Clinics* **2007,** *45* (2), 27-37.
75. Akdis, M.; Burgler, S.; Crameri, R.; Eiwegger, T.; Fujita, H.; Gomez, E.; Klunker, S.; Meyer, N.; O'Mahony, L.; Palomares, O.; Rhyner, C.; Ouaked, N.; Schaffartzik, A.; Van De Veen, W.; Zeller, S.; Zimmermann, M.; Akdis, C. A., Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. *J Allergy Clin Immunol* **2011,** *127* (3), 701-21.e1-70.
76. Petes, C.; Mariani, M. K.; Yang, Y.; Grandvaux, N.; Gee, K., Interleukin (IL)-6 Inhibits IL-27- and IL-30-Mediated Inflammatory Responses in Human Monocytes. *Front Immunol* **2018,** *9*, 256.
77. Klein, M. A.; Möller, J. C.; Jones, L. L.; Bluethmann, H.; Kreutzberg, G. W.; Raivich, G., Impaired neuroglial activation in interleukin-6 deficient mice. *Glia* **1997,** *19* (3), 227-33.

78. Chauhan, V. S.; Sterka, D. G.; Gray, D. L.; Bost, K. L.; Marriott, I., Neurogenic exacerbation of microglial and astrocyte responses to Neisseria meningitidis and Borrelia burgdorferi. *J Immunol* **2008,** *180* (12), 8241-8249.
79. Furr, S. R.; Moerdyk-Schauwecker, M.; Grdzelishvili, V. Z.; Marriott, I., RIG-I mediates nonsegmented negative-sense RNA virus-induced inflammatory immune responses of primary human astrocytes. *Glia* **2010,** *58* (13), 1620-1629.
80. Dobrovolskaia, M. A.; McNeil, S. E. J. E. o. o. b. t., Immunological and hematological toxicities challenging clinical translation of nucleic acid-based therapeutics. **2015,** *15* (7), 1023-1048.
81. Yao, W.; Mei, C.; Nan, X.; Hui, L., Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. *Gene* **2016,** *590* (1), 142-8.
82. Lee, Y.; Urban, J. H.; Xu, L.; Sullenger, B. A.; Lee, J., 2'Fluoro Modification Differentially Modulates the Ability of RNAs to Activate Pattern Recognition Receptors. *Nucleic Acid Ther* **2016,** *26* (3), 173-182.
83. Scheel, B.; Braedel, S.; Probst, J.; Carralot, J. P.; Wagner, H.; Schild, H.; Jung, G.; Rammensee, H. G.; Pascolo, S. J. E. j. o. i., Immunostimulating capacities of stabilized RNA molecules. **2004,** *34* (2), 537-547.
84. Hoerr, I.; Von Der Mulbe, F.; Pascolo, S., Immunostimulation by chemically modified RNA. Google Patents: 2005.
85. Chiu, Y. L.; Rana, T. M., RNAi in human cells: basic structural and functional features of small interfering RNA. *Molecular cell* **2002,** *10* (3), 549-61.
86. Eberle, F.; Giessler, K.; Deck, C.; Heeg, K.; Peter, M.; Richert, C.; Dalpke, A. H. J. T. J. o. I., Modifications in small interfering RNA that separate immunostimulation from RNA interference. **2008,** *180* (5), 3229-3237.
87. Sioud, M., Single-stranded small interfering RNA are more immunostimulatory than their double-stranded counterparts: A central role for 2′-hydroxyl uridines in immune responses. *European Journal of Immunology* **2006,** *36* (5), 1222-1230.
88. Sioud, M.; Furset, G.; Cekaite, L., Suppression of immunostimulatory siRNA-driven innate immune activation by 2′-modified RNAs. *Biochemical and Biophysical Research Communications* **2007,** *361* (1), 122-126.
89. Robbins, M.; Judge, A.; Liang, L.; McClintock, K.; Yaworski, E.; MacLachlan, I., 2'-O-methyl-modified RNAs act as TLR7 antagonists. *Mol Ther* **2007,** *15* (9), 1663-9.
90. Judge, A. D.; Bola, G.; Lee, A. C. H.; Maclachlan, I., Design of Noninflammatory Synthetic siRNA Mediating Potent Gene Silencing in Vivo. *Molecular Therapy* **2006,** *13* (3), 494-505.
91. Nguyen, D. N.; Chen, S. C.; Lu, J.; Goldberg, M.; Kim, P.; Sprague, A.; Novobrantseva, T.; Sherman, J.; Shulga-Morskaya, S.; De Fougerolles, A. J. M. T., Drug delivery–mediated control of RNA immunostimulation. **2009,** *17* (9), 1555-1562.
92. Avila, Y. I.; Chandler, M.; Cedrone, E.; Newton, H. S.; Richardson, M.; Xu, J.; Clogston, J. D.; Liptrott, N. J.; Afonin, K. A.; Dobrovolskaia, M. A., Induction of Cytokines by Nucleic Acid Nanoparticles (NANPs) Depends on the Type of Delivery Carrier. *Molecules (Basel, Switzerland)* **2021,** *26* (3), 652.
93. Halman, J. R.; Kim, K.-T.; Gwak, S.-J.; Pace, R.; Johnson, M. B.; Chandler, M. R.; Rackley, L.; Viard, M.; Marriott, I.; Lee, J. S. J. N. N., Biology; Medicine, A cationic amphiphilic co-polymer as a carrier of nucleic acid nanoparticles (Nanps) for controlled gene silencing, immunostimulation, and biodistribution. **2020,** *23*, 102094.

94. Kim, T.; Viard, M.; Afonin, K. A.; Gupta, K.; Popov, M.; Salotti, J.; Johnson, P. F.; Linder, C.; Heldman, E.; Shapiro, B. A., Characterization of Cationic Bolaamphiphile Vesicles for siRNA Delivery into Tumors and Brain. *Molecular therapy. Nucleic acids* **2020,** *20*, 359-372.
95. Kim, T.; Afonin, K. A.; Viard, M.; Koyfman, A. Y.; Sparks, S.; Heldman, E.; Grinberg, S.; Linder, C.; Blumenthal, R. P.; Shapiro, B. A., In Silico, In Vitro, and In Vivo Studies Indicate the Potential Use of Bolaamphiphiles for Therapeutic siRNAs Delivery. *Molecular therapy. Nucleic acids* **2013,** *2*, e80.
96. Nordmeier, S.; Ke, W.; Afonin, K. A.; Portnoy, V., Exosome mediated delivery of functional nucleic acid nanoparticles (NANPs). *Nanomedicine: Nanotechnology, Biology and Medicine* **2020,** *30*, 102285.
97. Bila, D.; Radwan, Y.; Dobrovolskaia, M. A.; Panigaj, M.; Afonin, K. A., The Recognition of and Reactions to Nucleic Acid Nanoparticles by Human Immune Cells. *Molecules (Basel, Switzerland)* **2021,** *26* (14), 4231.
98. Gupta, K.; Afonin, K. A.; Viard, M.; Herrero, V.; Kasprzak, W.; Kagiampakis, I.; Kim, T.; Koyfman, A. Y.; Puri, A.; Stepler, M.; Sappe, A.; KewalRamani, V. N.; Grinberg, S.; Linder, C.; Heldman, E.; Blumenthal, R.; Shapiro, B. A., Bolaamphiphiles as carriers for siRNA delivery: From chemical syntheses to practical applications. *Journal of controlled release : official journal of the Controlled Release Society* **2015,** *213*, 142-151.
99. Yu, J. Y.; Deruiter, S. L.; Turner, D. L., RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proceedings of the National Academy of Sciences* **2002,** *99* (9), 6047-6052.
100. Gitlin, L.; Karelsky, S.; Andino, R., Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **2002,** *418* (6896), 430-434.
101. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T., Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **2001,** *411* (6836), 494-498.
102. Dalby, B., Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods (San Diego, Calif.)* **2004,** *33* (2), 95-103.
103. Petros, R. A.; DeSimone, J. M., Strategies in the design of nanoparticles for therapeutic applications. *Nature Reviews Drug Discovery* **2010,** *9* (8), 615-627.
104. Johnson, M. B.; Halman, J. R.; Satterwhite, E.; Zakharov, A. V.; Bui, M. N.; Benkato, K.; Goldsworthy, V.; Kim, T.; Hong, E.; Dobrovolskaia, M. A. J. S., Programmable nucleic acid based polygons with controlled neuroimmunomodulatory properties for predictive QSAR modeling. **2017,** *13* (42), 1701255.
105. Canna, S. W.; Behrens, E. M., Making Sense of the Cytokine Storm: A Conceptual Framework for Understanding, Diagnosing, and Treating Hemophagocytic Syndromes. *Pediatric Clinics of North America* **2012,** *59* (2), 329-344.
106. Lenz, H. J., Management and Preparedness for Infusion and Hypersensitivity Reactions. *The Oncologist* **2007,** *12* (5), 601-609.
107. Dobrovolskaia, M. A., Pre-clinical immunotoxicity studies of nanotechnology-formulated drugs: Challenges, considerations and strategy. *Journal of Controlled Release* **2015,** *220*, 571-583.
108. Szebeni, J.; Simberg, D.; González-Fernández, Á.; Barenholz, Y.; Dobrovolskaia, M. A., Roadmap and strategy for overcoming infusion reactions to nanomedicines. *Nature nanotechnology* **2018,** *13* (12), 1100-1108.

109. Johnson, M. B.; Halman, J. R.; Satterwhite, E.; Zakharov, A. V.; Bui, M. N.; Benkato, K.; Goldsworthy, V.; Kim, T.; Hong, E.; Dobrovolskaia, M. A.; Khisamutdinov, E. F.; Marriott, I.; Afonin, K. A., Programmable Nucleic Acid Based Polygons with Controlled Neuroimmunomodulatory Properties for Predictive QSAR Modeling. *Small (Weinheim an der Bergstrasse, Germany)* **2017,** *13* (42).
110. Hong, E.; Halman, J. R.; Shah, A. B.; Khisamutdinov, E. F.; Dobrovolskaia, M. A.; Afonin, K. A., Structure and Composition Define Immunorecognition of Nucleic Acid Nanoparticles. *Nano letters* **2018,** *18* (7), 4309-4321.
111. Guo, S.; Li, H.; Ma, M.; Fu, J.; Dong, Y.; Guo, P. J. M. T.-N. A., Size, shape, and sequence-dependent immunogenicity of RNA nanoparticles. **2017,** *9*, 399-408.
112. Khisamutdinov, E. F.; Li, H.; Jasinski, D. L.; Chen, J.; Fu, J.; Guo, P. J. N. a. r., Enhancing immunomodulation on innate immunity by shape transition among RNA triangle, square and pentagon nanovehicles. **2014,** *42* (15), 9996-10004.
113. Halman, J. R.; Satterwhite, E.; Roark, B.; Chandler, M.; Viard, M.; Ivanina, A.; Bindewald, E.; Kasprzak, W. K.; Panigaj, M.; Bui, M. N.; Lu, J. S.; Miller, J.; Khisamutdinov, E. F.; Shapiro, B. A.; Dobrovolskaia, M. A.; Afonin, K. A., Functionally-interdependent shape-switching nanoparticles with controllable properties. *Nucleic acids research* **2017,** *45* (4), 2210-2220.
114. Afonin, K. A.; Viard, M.; Kagiampakis, I.; Case, C. L.; Dobrovolskaia, M. A.; Hofmann, J.; Vrzak, A.; Kireeva, M.; Kasprzak, W. K.; KewalRamani, V. N.; Shapiro, B. A., Triggering of RNA interference with RNA-RNA, RNA-DNA, and DNA-RNA nanoparticles. *ACS nano* **2015,** *9* (1), 251-9.
115. Esen, N.; Kielian, T., Central Role for MyD88 in the Responses of Microglia to Pathogen-Associated Molecular Patterns. *The Journal of Immunology* **2006,** *176* (11), 6802.

APPENDIX A

A.1 DNA cube sequences, A-F

DNA	Sequence (5'→3')
A	GGCAACTTTGATCCCTCGGTTTAGCGCCGGCCTTTTCTCCCACACTTTCACG
B	GGGAAATTTCGTGGTAGGTTTTGTTGCCCGTGTTTCTACGATTACTTTGGTC
C	GGACATTTTCGAGACAGCATTTTTTCCCGACCTTTGCGGATTGTATTTTAGG
D	GGCGCTTTTGACCTTCTGCTTTATGTCCCCTATTTCTTAATGACTTTTGGCC
E	GGGAGATTTAGTCATTAAGTTTTACAATCCGCTTTGTAATCGTAGTTTGTGT
F	GGGATCTTTACCTACCACGTTTTGCTGTCTCGTTTGCAGAAGGTCTTTCCGA

A.2 RNA cube sequences, A-F

RNA	Sequence (5'→3')
A	GGCAACUUUGAUCCCUCGGUUUAGCGCCGGCCUUUUCUCCCACACUUUCACG
B	GGGAAAUUUCGUGGUAGGUUUUGUUGCCCGUGUUUCUACGAUUACUUUGGUC
C	GGACAUUUUCGAGACAGCAUUUUUUCCCGACCUUUGCGGAUUGUAUUUUAGG
D	GGCGCUUUUGACCUUCUGCUUUAUGUCCCCUAUUUCUUAAUGACUUUUGGCC
E	GGGAGAUUUAGUCAUUAAGUUUUACAAUCCGCUUUGUAAUCGUAGUUUGUGU
F	GGGAUCUUUACCUACCACGUUUUGCUGUCUCGUUUGCAGAAGGUCUUUCCGA

A.3 A hybrid panel chart lists the properties for each cube organized by cube number and mixed ratio groups. Light red cells within the table represent samples with statistically significant IL-6 values compared to controls ($p<0.05$). Dark red cells located on the outer edges of the table indicate cubes that elicited statistically significant IFN-β levels

compared to controls ($p<0.05$).

						DNA	RNA						
Melting Temperature (°C)	Number of DNA Bases	Number of RNA Bases	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	6 : 0	0 : 6	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	Number of DNA Bases	Number of RNA Bases	Melting Temperature (°C)
35.9	312	0	12	0	0	1 ABCDEF	64 ABCDEF	0	0	12	0	312	59.8
Melting Temperature (°C)	Number of DNA Bases	Number of RNA Bases	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	5 : 1	1 : 5	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	Number of DNA Bases	Number of RNA Bases	Melting Temperature (°C)
37.1	260	52	8	4	0	2 ABCDEF	58 ABCDEF	0	4	8	52	260	56.6
37.1	260	52	8	4	0	3 ABCDEF	59 ABCDEF	0	4	8	52	260	56.6
37.1	260	52	8	4	0	4 ABCDEF	60 ABCDEF	0	4	8	52	260	56.6
37.1	260	52	8	4	0	5 ABCDEF	61 ABCDEF	0	4	8	52	260	56.6
37.1	260	52	8	4	0	6 ABCDEF	62 ABCDEF	0	4	8	52	260	56.6
37.1	260	52	8	4	0	7 ABCDEF	63 ABCDEF	0	4	8	52	260	56.6
Melting Temperature (°C)	Number of DNA Bases	Number of RNA Bases	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	4 : 2	2 : 4	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	Number of DNA Bases	Number of RNA Bases	Melting Temperature (°C)
39.9	208	104	5	6	1	8 ABCDEF	43 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	4	8	0	9 ABCDEF	44 ABCDEF	0	8	4	104	208	49.8
39.9	208	104	5	6	1	10 ABCDEF	45 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	11 ABCDEF	46 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	12 ABCDEF	47 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	13 ABCDEF	48 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	4	8	0	14 ABCDEF	49 ABCDEF	0	8	4	104	208	49.8
39.9	208	104	5	6	1	15 ABCDEF	50 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	16 ABCDEF	51 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	17 ABCDEF	52 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	18 ABCDEF	53 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	19 ABCDEF	54 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	20 ABCDEF	55 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	5	6	1	21 ABCDEF	56 ABCDEF	1	6	5	104	208	49.8
39.9	208	104	4	8	0	22 ABCDEF	57 ABCDEF	0	8	4	104	208	49.8
Melting Temperature (°C)	Number of DNA Bases	Number of RNA Bases	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	3 : 3	3 : 3	Number of DNA Helices	Number of Hybrid Helices	Number of RNA Helices	Number of DNA Bases	Number of RNA Bases	Melting Temperature (°C)
40.7	156	156	2	8	2	23 ABCDEF	33 ABCDEF	2	8	2	156	156	40.7
40.7	156	156	2	8	2	24 ABCDEF	34 ABCDEF	2	8	2	156	156	40.7
40.7	156	156	3	6	3	25 ABCDEF	35 ABCDEF	3	6	3	156	156	40.7
40.7	156	156	3	6	3	26 ABCDEF	36 ABCDEF	3	6	3	156	156	40.7
40.7	156	156	2	8	2	27 ABCDEF	37 ABCDEF	2	8	2	156	156	40.7
40.7	156	156	2	8	2	28 ABCDEF	38 ABCDEF	2	8	2	156	156	40.7
40.7	156	156	2	8	2	29 ABCDEF	39 ABCDEF	2	8	2	156	156	40.7
40.7	156	156	3	6	3	30 ABCDEF	40 ABCDEF	3	6	3	156	156	40.7
40.7	156	156	3	6	3	31 ABCDEF	41 ABCDEF	3	6	3	156	156	40.7
40.7	156	156	2	8	2	32 ABCDEF	42 ABCDEF	2	8	2	156	156	40.7