ARTICLE @ VIROLOGY

Novel Function of Nucleic Acid Substances as Viral Vaccine Adjuvants to Enhance Immune Response

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ABSTRACT

In recent years, nucleic acid-based substances have garnered significant attention in immunology as vaccine adjuvants. With a deeper understanding of the immune system, researchers have increasingly recognized the important role of nucleic acid-based substances (such as CpG oligonucleotides and polyI:C) in enhancing vaccine-induced immune responses. This article reviews the mechanisms by which nucleic acid adjuvants promote antigen presentation and T-cell activation through the stimulation of specific immune pathways, as well as the clinical applications of nucleic acid adjuvants in developing vaccines against infectious diseases. Future research on nucleic acid adjuvants should focus on aspects such as stability, safety, and exploring adjuvant combinations. Currently, no corresponding vaccine products have been approved for marketing, but some nucleic acid-adjuvanted vaccines have yet been approved for commercial use, several candidates have advanced to human clinical trials. In conclusion, research on nucleic acid-based vaccine adjuvants is rapidly progressing. Their unique immune activation mechanisms and broad clinical potential offer promising new opportunities for vaccine research and development.

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Abbreviations: DCs, Dendritic Cells; PRRs, Pattern Recognition Receptors; TLRs, Toll-like Receptors; dsRNA, double-stranded RNA; RLR，RIG-I-like Receptor ; BCG, Bacillus Calmette-Guérin; BCG-PSN, BCG Polysaccharide Nucleic Acid; APCs, Antigen-Presenting Cells; siRNA, small interfering RN; VZV, Varicella-Zoster Virus.

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Introduction

Vaccine adjuvants are substances that can enhance the body's immune response when administered alongside antigens, thereby improving vaccine efficacy. Adjuvants can be classified into two main categories: immunostimulants and delivery systems. Their primary mechanisms of action include the following: first, adjuvants can modify the physical properties of antigens, making them more easily recognized by the immune system. Second, they activate immune cells, such as dendritic cells (DCs), enhancing their ability to uptake, process, and present antigens. Finally, adjuvants can modulate both the type and magnitude of the immune response, resulting in a more durable and effective immunity[1].

In recent years, significant progress has been made in research on nucleic acid-based substances as vaccine adjuvants. With a deeper understanding of the immune system, particularly the innate immune system, researchers have discovered that nucleic acid adjuvants can effectively activate immune responses, making them a crucial component of vaccine development. These adjuvants induce robust immune responses by activating pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), thereby enhancing the immunological efficacy of vaccines[2].

The mechanisms of nucleic acid adjuvants primarily depend on their interactions with immune cells. For example, synthetic nucleic acid-based adjuvants, such as CpG oligonucleotides and poly(I:C), can significantly enhance the immunological efficacy of vaccines by promoting cytokine release and strengthening the activation of B cells and T cells[3]. These nucleic acid adjuvants not only function as potentiators in vaccines but also serve as independent imm--unotherapeutic agents, aiding in the control of tumors and infectious diseases [4-6]. The structural diagrams of currently common vaccine adjuvants are shown in Figure 1.

Future research will focus on optimizing the design and application of nucleic acid adjuvants, including improving their stability through molecular modifications and deve--loping combination adjuvants. Researchers aim to enhance the stability and bioavailab--ility of nucleic acid adjuvants while minimizing potential immune-related side effects [7]. Additionally, investigating the synergistic effects between various nucleic acid adjuvants and other immunomodulators will offer new insights and strategies for vaccine development [8].

Classification and Immune-Enhancing Mechanisms of Nucleic Acid Adjuvants

Research on nucleic acid-based substances as vaccine adjuvants has increasingly become a focal point in vaccine development. The primary types of nucleic acid adjuvants include CpG oligonucleotides, double-

-stranded RNAs (dsRNAs), such as polyinosinic-polycytidylic acid (poly I:C), and other nucleic acid adjuvants, including small interfering RNAs (siRNAs). These nucleic acid adjuvants enhance vaccine efficacy by activating the host immune system and thereby strengthening the immune response.

1.Types of Nucleic Acid-Based Vaccine Adjuvants

1.1 CpG Oligonucleotides

CpG oligonucleotides are synthetic sequences containing unmethylated cytosine--guanine dinucleotides (CpG) that activate the immune system through Toll-like receptor 9 (TLR9).

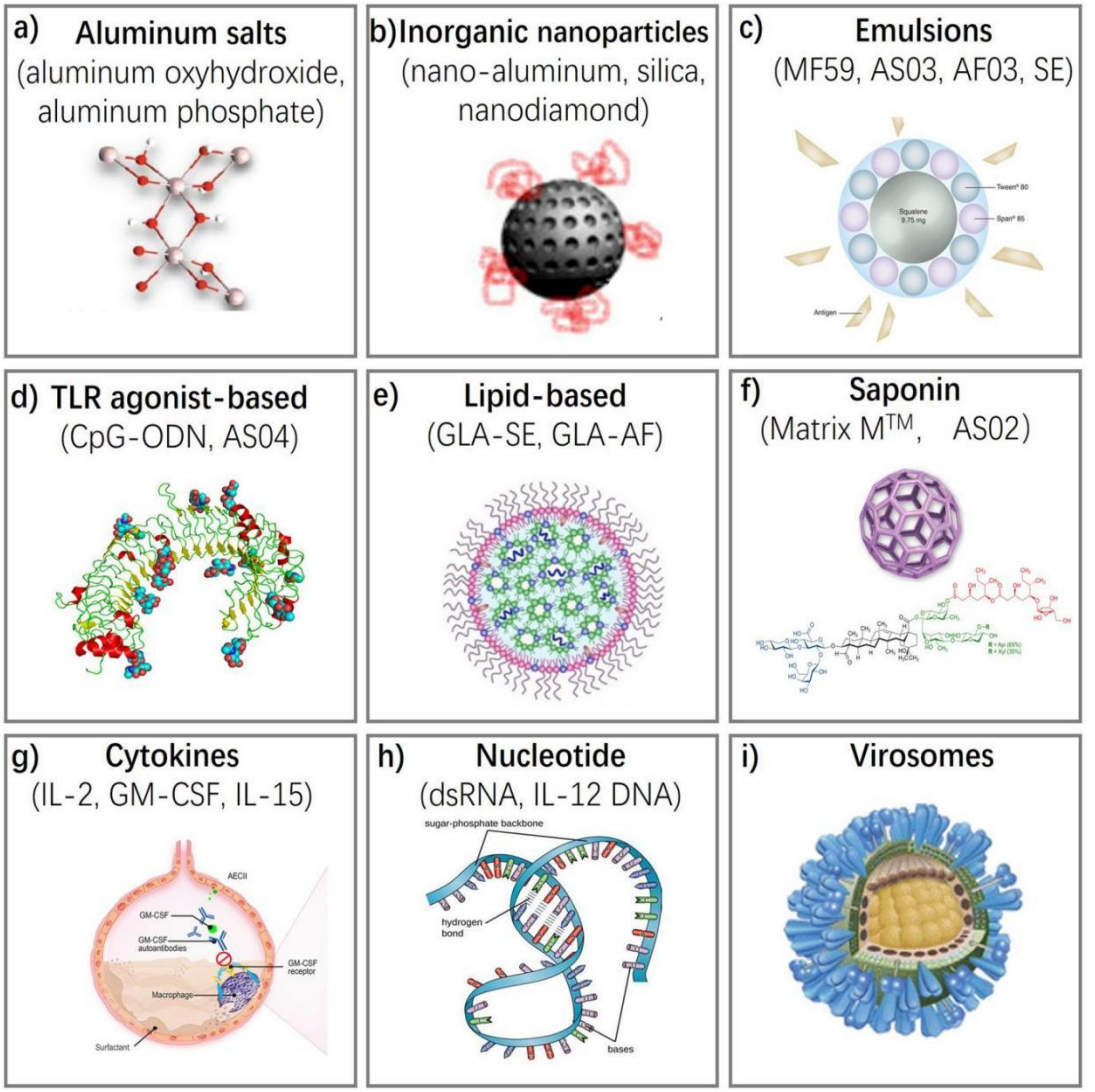

Fig. 1: Schematic diagram of common vaccine adjuvants.

Their structural features allow them to specifically recognize and bind to TLR9, thereby inducing strong immune responses, including cytokine production and immune cell activation [9].

Studies have demonstrated that CpG oligonucleotides with different sequences exhibit significant variations in their immunomodulatory functions; certain CpG ODNs can markedly enhance the proliferation and activation of B cells [10].

Furthermore, the application of CpG oligonucleotides in cancer vaccines has shown promising efficacy, as they promote tumor-specific immune responses and inhibit tumor growth [11].

1.2 Double-Stranded RNA

Double-stranded RNA (dsRNA) is an intermediate in viral replication and serves as a marker of infection. It is recognized by Toll-like receptor 3 (TLR3), two members of the RIG-I-like receptor (RLR) family-namely, the RNA helicase RIG-I and melanoma differentiation-associated gene 5 (MDA-5), and the NLR pyrin domain-containing 3 (NLRP3) protein of the NLR family. These proteins trigger the release of inflammatory cytokines, thereby activating innate immunity, which subsequently elicits adaptive immune responses [12]. The primary cytokines involved in this response are type I interferons, including IFN-α and IFN-β [13].

As adjuvants, dsRNAs are mostly artificially synthesized, with common examples including poly(I:C), poly(I:C12U), poly ICLC, and poly(A:U) [14].

Polyinosinic-polycytidylic acid (poly(I:C)) is a synthetic double-stranded RNA molecule that induces immune responses by activating TLR3 and RLRs. Its primary mechanisms involve triggering antiviral and pro--inflammatory immune responses, thereby enhancing the host's resistance to pathogens [15]. Studies have demonstrated that poly(I:C) effectively activates DCs, promoting their maturation and enhancing their antigen--presenting capabilities [16].

To improve the stability of poly(I:C), covalent conjugation with kanamycin or polylysine can be employed, resulting in the formation of pika adjuvant (kanamycin) [17] and EP adjuvant (polylysine) [18]. These modified poly(I:C) adjuvants exhibit increased resistance to RNase hydrolysis, along with enhanced stability and immuno--stimulatory activity.

Although dsRNAs, including poly(I:C), are promising adjuvant candidates, several challenges remain to be addressed. Most notably, due to technical limitations in the manufacturing process, poly(I:C) is inherently heterogeneous[19]. This hetero--geneity complicates the determination of its exact size, molecular weight, and structure, while the biological activity of dsRNA depends on its molecular size. Additionally, batch-to-batch variations in the pharmaco--logical effects of poly(I:C) have been observed, and its heterogeneity is also linked to toxic effects [20, 21].

Commercially available poly(I:C) and its derivatives exhibit significant heterogeneity, resulting in inconsistencies between batches [22]. From a production standpoint, multiple heating and slow cooling steps are required to re-anneal poly-I and poly-C chains, making reconstitution challenging. By employing polymerase chain reaction (PCR)-coupled bidirectional in vitro transcription (IVT) technology, researchers have developed homogeneous and quality-controllable next-generation dsRNA TLR3 agonists (NexaVant, NVT), which are anticipated to represent the next generation of modified poly(I:C) adjuvants [23].

1.3 BCG Polysaccharide Nucleic Acid (BCG-PSN)

BCG-PSN is a polysaccharide-nucleic acid complex extracted from Bacillus Calmette-Guérin (BCG) using the hot phenol method. It functions by activating macrophages and enhancing both T cell- and B cell-mediated cellular and humoral immune responses. As an immunomodulator, BCG-PSN induces Th1-mediated immune responses by promoting T cell activation and differentiation into Th1 subsets, as well as activating Toll-like receptor (TLR) signaling pathways. This process facilitates the secretion of cytokines such as IL-2 and IFN-γ [24].

As a vaccine adjuvant, its mechanism of action involves recognition by PRRs, thereby initiating innate immunity. It activates immature dendritic cells (DCs), promoting their differentiation into antigen--presenting cells (APCs). Subsequently, MHC I and MHC II molecules on the surface of APCs bind to immature CD8+ and CD4+ lymphocytes, inducing the proliferation and differentiation of T lymphocytes to elicit adaptive immunity [25].

1.4 Other Nucleic Acid Adjuvants (e.g., siRNA)

Other nucleic acid adjuvants, such as small interfering RNA (siRNA), have also shown potential as vaccine adjuvants. siRNA primarily regulates immune responses by silencing the expression of specific genes, exhibiting high specificity and controllability, making it suitable for vaccine development against cancer and viral infections [5, 26].

Studies have demonstrated that when siRNAs are combined with other immune activators, they can significantly enhance the immunogenicity and therapeutic efficacy of vaccines, offering new insights and directions for future vaccine development [11].

2. Immunopotentiating Mechanisms of Nucleic Acid Adjuvants

2.1 TLR-Mediated Immune Responses

TLRs are essential pattern recognition receptors in the innate immune system that detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Activation of TLRs initiates a cascade of immune responses, including cytokine release and the activation of APCs. Nucleic acid-based

adjuvants, such as CpG oligonucleotides and poly(I:C), bind to TLRs to trigger downstream signaling pathways, promoting the production of interferons and other cytokines, thereby enhancing specific immune responses [27, 28].

For instance, CpG oligodeoxynucleotides (ODN) stimulate B cell proliferation and antibody production via TLR9, while poly(I:C) activates dendritic cells through TLR3, augmenting their antigen-presenting capacity [4, 29]. This TLR-mediated immune response not only enhances vaccine immunogenicity but also plays a critical role in antiviral and antitumor immunity [6, 15].

2.2 RLRs Signaling Pathway

RIG-I-like receptors (RLRs) are a crucial class of intracellular pattern recognition receptors primarily responsible for detecting viral RNA in the cytoplasm. Upon binding to viral RNA, RIG-I activates the downstream mitochondrial antiviral signaling protein (MAVS), thereby initiating interferon production and antiviral immune responses [30, 31].

In numerous studies, activation of RIG-I-like receptors (RLRs) has been shown to enhance cellular resistance to viruses. For instance, RIG-I activation induces the production of antiviral cytokines, such as interferons α and β, which play a crucial role in antiviral immunity [32].

Furthermore, regulation of the RLR signaling pathway is closely associated with the onset and progression of various diseases. Researchers are investigating the potential to improve vaccine efficacy by modulating the RLR signaling pathway [33, 34].

2.3 Activation of Inflammasomes and Cytokine Release

Inflammasomes are intracellular multi--protein complexes that detect intracellular pathogens and damage signals, thereby triggering inflammatory responses. Their activation is typically accompanied by the release of cytokines, particularly IL-1β and IL-18, which play crucial roles in regulating immune responses and inflammation [35, 36].

Nucleic acid-based adjuvants can enhance immune responses by activating inflamma--somes, thereby promoting cytokine release. For example, studies have demonstrated that nucleic acid adjuvants such as poly(I:C) can activate the NLRP3 inflammasome, induce the production of IL-1β, and subsequently enhance antiviral and antitumor immunity [37].

Furthermore, inflammasome activation is implicated in the pathogenesis of various diseases, and researchers are investigating strategies to improve therapeutic efficacy by modulating inflammasome activity [36, 38].

Application of Nucleic Acid Adjuvants in Viral Vaccines

The advantage of recombinant subunit vaccines lies in their safety and stability under refrigerated conditions; however, due to their low immunogenicity, the use of

adjuvants is essential in their development. Additionally, adjuvants are also necessary for inactivated whole-virus vaccines. The application of nucleic acid adjuvants in vaccines for viral infectious diseases has garnered increasing attention, particularly in the development of vaccines against influenza, COVID-19, rabies, herpes zoster, and others.

1. Influenza Vaccines

Although current influenza vaccines demonstrate some immunogenicity, the antibody levels induced in the population remain relatively low, failing to achieve the desired efficacy. Consequently, influenza continues to pose a significant threat to public health.

Adjuvants are substances added to vaccines to enhance immune responses to antigens by improving antigen immunogen--icity, accelerating vaccine-induced responses, facilitating immunoregul- -ation, and enhancing vaccine stability [39].

Alum adjuvants are the most commonly used in influenza vaccines. In addition to alum, various other adjuvants, such as MF59 and AS03, are also employed in the clinical administration of influenza vaccines [40]. However, traditional adjuvants have limitations, including the potential to induce fever and headaches, disrupt cellular metabolism, and trigger autoimmune diseases [41].

Therefore, the development of safe and effective novel adjuvants is essential. A recent study using a mouse model demonstrated that BCG polysaccharide nucleic acid (BCG-PSN) is a promising candidate as a vaccine adjuvant [43].

2. COVID-19 Vaccines

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has affected a vast population worldwide. In March 2020, the World Health Organization declared it a pandemic, and since then, it has resulted in extensive morbidity and mortality globally. Vaccines are a crucial tool for controlling the current pandemic, and research on COVID-19 therapies is progressing at an unprecedented pace.

Vaccine platforms are categorized into two groups: those based on viral components and those based on the whole virus. Viral component-based vaccines include protein subunits, virus-like particles, DNA-based vaccines, RNA-based vaccines, non--replicating viral vectors, and replicating viral vectors.

In research on COVID-19 vaccines, a recombinant protein vaccine comprising a recombinant SARS-CoV-2 spike protein trimer expressed in CHO cells, combined with a CpG7909/aluminum hydroxide composite adjuvant, was evaluated for its immunogenicity. Studies demonstrated that it induced robust neutralizing antibody responses and significant CD4+ T cell responses in both mice and non-human primates. Vaccine-induced neutralizing

antibodies remained at high levels for at least six months. Challenge studies showed that the vaccine significantly reduced viral load and inflammation in the lungs of golden Syrian hamsters infected with SARS-CoV-2 [44]. Additionally, vaccines using only CpG adjuvants have been developed, such as those employing CpG 684 as an adjuvant for inactivated COVID-19 vaccines [45].

Furthermore, researchers have utilized a ternary composite adjuvant-aluminum /cGAMP/poly(I:C)-which demonstrated a significant synergistic effect, rapidly eliciting robust immune responses. The combination of this ternary adjuvant system with the S1 protein represents a promising COVID-19 vaccine candidate [46].

3. Rabies Vaccines

Rabies is a fatal zoonotic disease with a 100% mortality rate once symptoms appear. Post-exposure vaccination against rabies is essentially a race against the virus; therefore, the earlier antibodies are produced following vaccination, the better the outcome [47].

Nucleic acid adjuvants have also shown promise in the development of rabies virus vaccines. Studies have demonstrated that vaccines combined with nucleic acid adjuvants can effectively induce strong immune responses and provide robust protective efficacy in animal models.

A rabies vaccine formulated with PIKA as an adjuvant was tested in mice infected with seven rabies virus strains prevalent in China and demonstrated a protective efficacy exceeding 80% without the need for immunoglobulin. The PIKA-adjuvanted rabies vaccine induced a more pronounced increase in neutralizing antibody levels as early as five days post-vaccination, surpassing the immune responses elicited by currently marketed inactivated, adjuvant-free rabies vaccines, particularly in terms of cellular immunity. Vaccination with the PIKA rabies vaccine significantly increased the number of IFN-γ-producing T cells targeting the antigen, accompanied by elevated levels of IL-1β, IL-6, CCL-2, and TNF-α at the injection site. Furthermore, increased serum levels of chemokines and proinflammatory molecules were observed following administration of the PIKA rabies vaccine [48].

Phase I and II clinical results indicated that the accelerated schedule of the PIKA rabies vaccine was well tolerated and demonstrated non-inferior immunogenicity compared to the conventional schedule using commercially available vaccines in healthy adults [49].

Multiple studies have demonstrated that the PIKA rabies vaccine has significant potential as a highly effective immunization method, substantially enhancing the efficacy of rabies vaccines. Currently, the PIKA rabies vaccine has received approval for Phase III clinical trials in China, and an improved PIKA adjuvant-based rabies vaccine (EP adjuvant rabies vaccine) is also under development [18, 48].

4. Herpes Zoster Vaccines

Varicella-zoster virus (VZV) is highly contagious, and nearly everyone is exposed to it before adulthood. Primary infection causes chickenpox. After spontaneous recovery, the virus is not eliminated but remains latent in sensory ganglia. It can reactivate when cellular immune responses decline, often due to aging, leading to herpes zoster [50]. Since antiviral drugs and pain relievers only alleviate some symptoms, vaccination has become the preferred strategy for managing VZV infections.

The currently available subunit herpes zoster vaccine, Shingrix, is superior to the attenuated vaccine Zostavax in both safety and efficacy [51]. However, its non--lyophilizable liposomal delivery system and the limited supply of the naturally derived immune adjuvant QS-21 still present challenges that require improvement. Researchers are also exploring the use of nucleic acid adjuvants to enhance vaccine immune efficacy.

A recombinant subunit vaccine targeting the varicella-zoster virus glycoprotein E, formulated with poly(lactic-co-glycolic acid) nanoparticles and nucleic acid adjuvants, has demonstrated strong immune efficacy in mice. Vaccines combined with nucleic acid adjuvants significantly improved the immune protection rate [29]. Additionally, the composite adjuvant Al-Poly(I:C), prepared from aluminum oxyhydroxide nanorods (AlOOH NRs) and Poly(I:C), enhanced both humoral and cellular immune responses in herpes zoster vaccines [52].

Conclusion and Future Directions

In recent years, nucleic acid-based substances such as DNA and RNA have demonstrated not only strong immune--activating capabilities in basic research but have also provided new insights into vaccine efficacy and safety. With advances in understanding immune mechanisms and the rational optimization of application protocols, nucleic acid-based substances are expected to become a crucial component of future vaccine development.

Their primary advantage lies in their ability to activate the host immune system, thereby enhancing vaccine-induced immune responses. This property enables researchers to design vaccines with greater efficacy to address the challenges posed by emerging infectious diseases.

Research on nucleic acid-based substances as vaccine adjuvants continues to face significant challenges related to safety and side-effect management. Although nucleic acid adjuvants, such as CpG oligonucleotides and poly(I:C), demonstrate excellent efficacy in enhancing immune responses, their associated inflammatory reactions and potential adverse effects warrant careful consideration. Studies have indicated that certain nucleic acid adjuvants can induce local or systemic adverse

reactions, which impede their clinical application. For example, poly(I:C) may provoke intense inflammatory responses in some cases, resulting in fever and other discomforts in patients. Consequently, the development of novel, non-inflammatory nucleic acid adjuvants has become a key research focus [15]. Future investigations should aim to optimize the structural design of nucleic acid adjuvants to minimize side effects while preserving their immuno--potentiating capabilities.

The progress and advantages of adjuvant combinations represent a central focus in current vaccine technology development, particularly in addressing emerging infectious diseases and enhancing vaccine efficacy. Nucleic acid adjuvants, such as CpG oligonucleotides and poly(I:C), can be combined with aluminum salts, lipid nanoparticles (LNPs), QS-21, squalene, and other components to form effective adjuvant systems.

the antimicrobial peptide KLK and the TLR9 agonist ODN1a. This adjuvant has demonstrated strong immunogenicity and protective efficacy in animal models, inducing antigen-specific humoral and cellular immune responses.

The EP adjuvant consists of aluminum salts, polycationic substances, and the TLR3 agonist poly(I:C), showing promising potential in terms of safety and efficacy [18]. Covalent bonds form between the -PO_3^- groups on the poly(I:C) backbone and the hydroxyl groups on AlOOH, enabling Al-Poly(I:C) to exhibit enhanced adjuvant activity and more balanced adaptive immune responses, as illustrated in Figure 2 [52]. The development of adjuvant combinations is evolving from single function approaches to multi-mechanism synergy, offering significant advantages in comprehensively improving vaccine efficacy, safety, and cost-effectiveness. This progress provides essential technical support for addressing future infectious disease challenges.

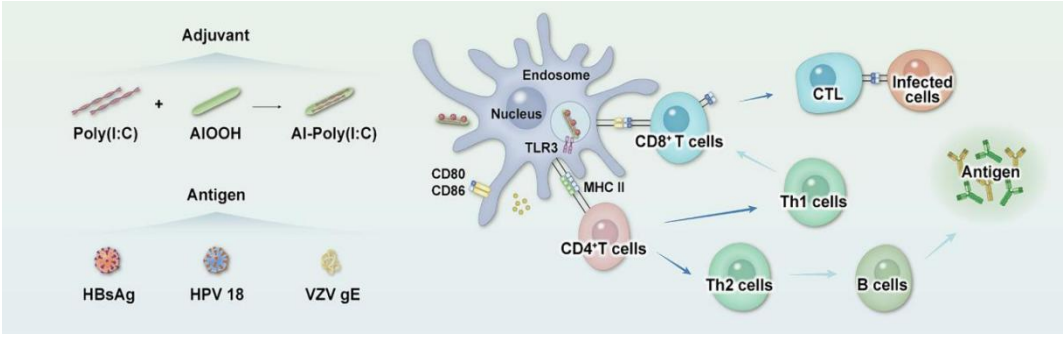

Figure 3: Mechanism of action of combined nucleic acid adjuvants in vaccines under development [52].

acid-based substances as vaccine adjuvants is progressing rapidly. Their unique immune activation mechanisms and broad clinical application potential have created new opportunities for vaccine development. With ongoing technological advancements and a deeper understanding of immune mechanisms, nucleic acid adjuvants are expected to play an increasingly important role in future vaccine research and development.

Competing interests

The authors declare all financial and non-financial competing interests.

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