

Engineering intramolecular adjuvants for next-generation vaccines

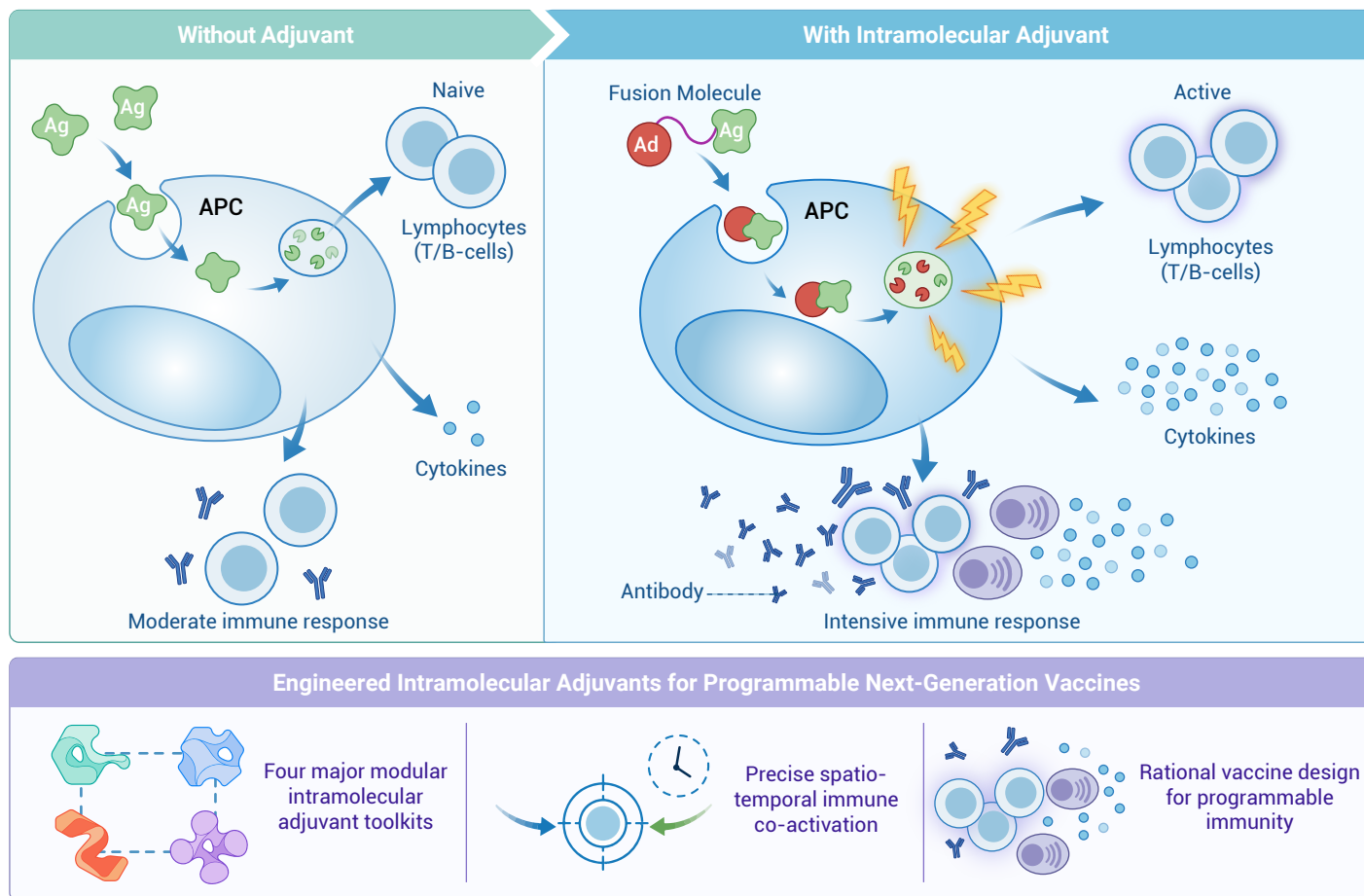
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Intramolecular adjuvants fuse adjuvants with antigens into one molecule for precise immune activation.
- Four major classifications: bacterial toxin derivatives, PRR ligands, cytokines and complement fragments.
- This toolkit enables programmable immunity for next-generation vaccine design.

Engineering intramolecular adjuvants for next-generation vaccines

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Vaccines play a pivotal role in reducing the global burden of infectious diseases. Currently, vaccine development is transitioning from empirical approaches toward precision engineering. The intramolecular adjuvant strategy, which fuses adjuvants and antigens into single molecules via genetic engineering, emerges as a promising strategy with the potential to play a key role in this transition. Through precise control of co-delivery and synergistic signaling, intramolecular adjuvants can modulate the magnitude, duration, and type of immune response, thereby advancing vaccine design toward a more programmable paradigm, in which immune activation can be rationally tuned at the molecular level, ultimately enabling “programmable immunity”. This review systematically summarizes the current toolbox of genetically encoded intramolecular adjuvants, including derivatives of bacterial toxins, pattern recognition receptor (PRR) ligands, cytokines, and the complement fragments. By integrating structural biology, synthetic biology, and immunology, we delineate their mechanisms in receptor-targeted delivery and synergistic immune pathway activation. Notably, artificial intelligence (AI) might accelerate the rational development of intramolecular adjuvants via sequence mining, structural optimization and de novo design. Collectively, this review aims to establish a systematic framework for intramolecular adjuvant selection and design, advancing a data-driven paradigm to counter emerging pathogens.

INTRODUCTION

Vaccines represent a key strategy for preventing both infectious and noncommunicable chronic diseases, with their technological evolution closely tied to progress in immunology and biotechnology. Advances in genomics, protein engineering, and bioinformatics, underpinned by genetic engineering, have transformed vaccine development through reverse vaccinology and structural vaccinology,¹ accelerating the progress of subunit, nucleic acid, and viral vector vaccine platforms. These approaches yield vaccines with well-defined molecular composition, high antigen purity, and enhanced biological safety, positioning them as leading candidates in next-generation vaccine design.² However, many modern vaccines, particularly subunit platforms, suffer from limited immunogenicity, often failing to adequately activate antigen-presenting cells (APCs) or induce protective T- and B-cell responses.^{3,4} Adjuvants serve as immune potentiators that enhance vaccine efficacy by prolonging antigen persistence, activating pattern recognition receptors (PRRs), promoting cytokine secretion, and so on. To date, several adjuvants have been clinically approved, including aluminum salts, AS04, and CpG ODNs.⁵

Immunogenicity, the ability of a substance to elicit an immune response, depends on the synergistic interplay between antigenicity and adjuvanticity.⁶ Optimal immunity requires precise spatiotemporal co-delivery of antigen and adjuvant, yet most current formulations rely on simple physical mixtures. A major limitation of this approach is the lack of controlled co-localization at the cellular level. In mixed formulations, antigens and adjuvants are not molecularly conjugated and are present in non-stoichiometric ratios, leading to stochastic uptake by APCs. As a result, APCs may take up antigen without adjuvant, or *vice versa*, compromising synergistic activation at the critical moment of immune initiation. Effective T- and B-cell activation requires APCs to receive two synchronous signals: Signal 1 (antigen presentation) and Signal 2 (co-stimulation).⁶ The random co-uptake inherent in mixed formulations often fails to ensure simultaneous signal delivery, reducing activation efficiency. To compensate, higher doses of antigen or adjuvant are often used, which increases the risk of adverse reactions while failing to trigger a

commensurate improvement in immunity.

Recent advances in molecular biology and genetic engineering have enabled the development of intramolecular adjuvants (also termed built-in adjuvants), where the adjuvant is integrated with the antigen into a single molecular entity. This strategy ensures precise co-delivery and represents a shift toward “programmable immunity”, an era in which immune responses can be pre-programmed in type, strength, and spatiotemporal dynamics with unprecedented precision. Current intramolecular adjuvant design primarily involves genetic fusion or chemical conjugation.^{7,8} Chemical conjugation covalently links the adjuvant to antigen, offering modularity while involving complex synthesis, heterogeneous products, and high costs. In contrast, genetic fusion employs cellular systems to produce homogeneous fusion proteins, providing a more scalable and structurally uniform alternative approach. By employing linker peptides and genetic code reprogramming, antigen-adjuvant fusion proteins achieve proper folding and functional domain orientation, enabling precise control over the timing and strength of immune activation. Moreover, genetically engineered intramolecular adjuvants can be constructed in various molecular forms, including DNA, RNA, and proteins, further expanding their versatility in vaccine design. Notably, for DNA and RNA vaccines, the encoded fusion protein is not directly administered. Instead, the nucleic acid itself must be delivered into host cells via specialized carriers, where it undergoes *in vivo* transcription and translation to produce the functional antigen-adjuvant fusion protein, thereby eliciting the desired immune response (Figure 1).

Genetically engineered intramolecular adjuvants enable antigen-adjuvant co-internalization by APCs, which creates spatial co-localization and markedly potentiates synergistic immune signaling. Notably, mere co-internalization cannot ensure the synchronous activation of immune signaling cascades. Such precise spatiotemporal coordination optimizes the stoichiometric and temporal-spatial synergy between Signal 1 and Signal 2. Beyond core signaling synergy, specific adjuvant modules such as Fc fusion domains can extend antigen half-life to achieve metabolic regulation of antigen exposure.^{9–11} Additionally, fused adjuvant moieties such as GM-CSF¹² and flagellin^{13,14} can directly engage specific immune cell receptors, enabling fine-tuning of immune response intensity and polarization. Notably, modern genetic engineering technologies have surpassed traditional linear fusion design strategies. When integrated with artificial intelligence (AI)-assisted protein design, advanced modification techniques such as codon optimization, glycosylation editing, and multimerization domain engineering are extensively utilized to strengthen the structural stability and immunogenicity of fusion constructs.¹⁵ The integration of genetic engineering and AI-driven design enables researchers to rationally screen and match intramolecular adjuvants based on antigen characteristics, rapidly refine fusion protein structures, and identify optimal vaccine formats. Apart from these core construction strategies, multiple emerging approaches are further expanding the design landscape of intramolecular adjuvant vaccines, including non-genetically encoded synthetic strategies. Numerous synthetic small-molecule adjuvants have entered clinical evaluation, including imidazopyrimidine TLR7/8 agonist PVP-037 and various STING agonists such as cyclic dinucleotides and non-nucleotide agonists.^{5,16,17}

This review systematically summarizes the mechanisms and recent advances of genetically engineered intramolecular adjuvants, providing a scientific basis for their rational selection in vaccine development. We focus on four major classes, including derivatives of bacterial toxins, PRR ligands, cytokines, and the complement fragments, demonstrating their structure-activity relationships and application potential. Nevertheless, their clinical

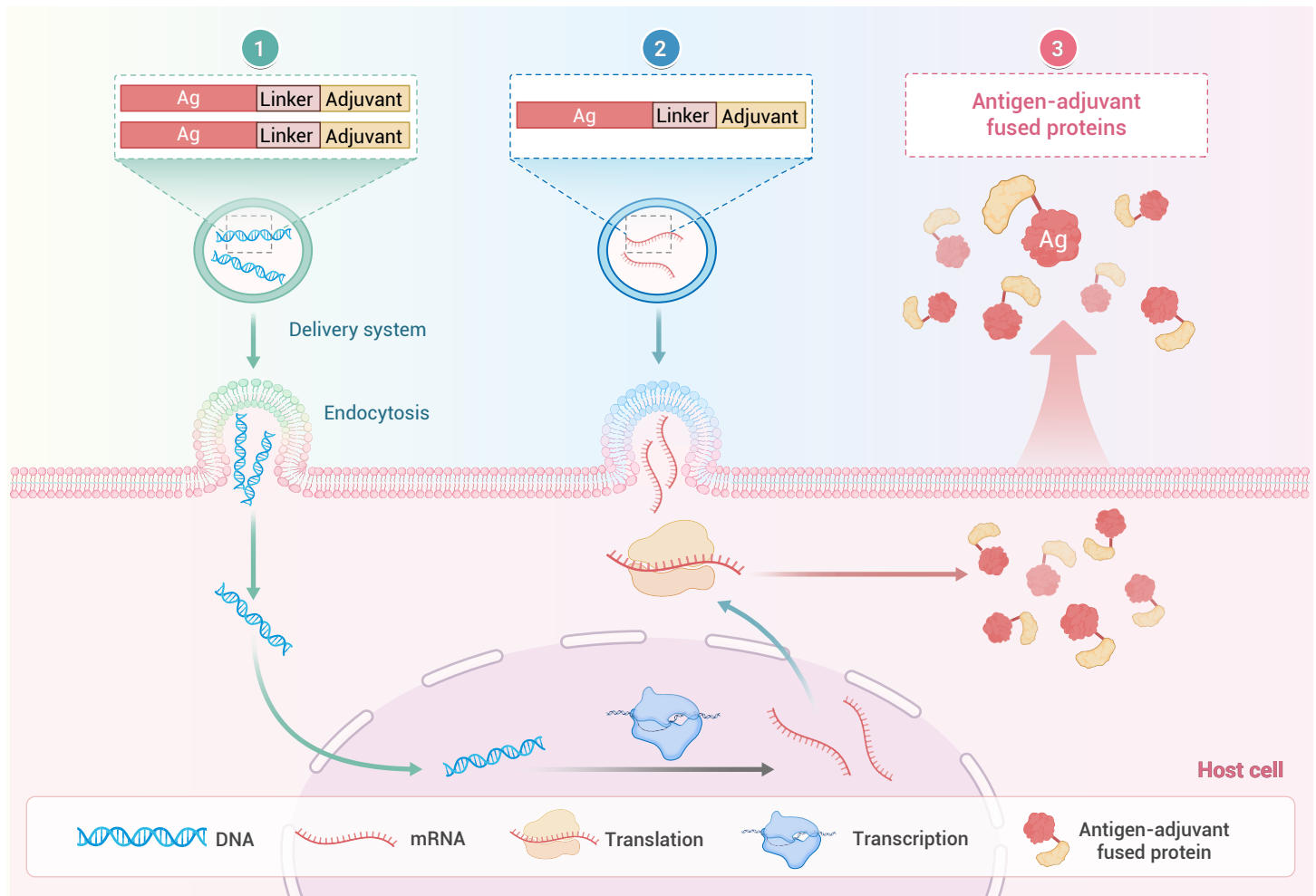


Figure 1. Schematic overview of intramolecular adjuvant–antigen fusion constructs and their delivery pathways Intramolecular adjuvants are genetically encoded as a single-chain fusion with the antigen, ensuring stoichiometric co-delivery and precise spatiotemporal co-localization in antigen-presenting cells. The schematic illustrates three representative gene-based vaccine platforms, including DNA, mRNA, and recombinant protein subunit vaccines, as well as their distinct routes for producing fusion proteins. For nucleic acid vaccines (DNA and mRNA), the fusion construct is delivered into host cells via transfection or lipid nanoparticles, where it is transcribed (DNA) or directly translated (mRNA); the host cell machinery subsequently produces and secretes the antigen–adjuvant fusion protein. In contrast, recombinant subunit vaccines are produced as purified fusion proteins in heterologous expression systems (e.g., bacteria, yeast, or mammalian cells) and administered as pre-formed immunostimulatory molecules. The modular domain organization highlights the flexible linker that preserves proper folding, the antigen moiety for specific epitope presentation, and the adjuvant domain responsible for targeting innate receptors and activating downstream signaling pathways.

translation faces several obstacles covering conformational integrity maintenance, immunological safety control and regulatory manufacturing compliance. Notably, AI holds great potential to tackle these bottlenecks, shifting the development paradigm from empirical trial-and-error to predictive rational design via high-throughput adjuvant sequence mining, structure-guided fusion optimization and *de novo* design of customized immune modules. Integrating structural biology, computational immunology and experimental data, this review delineates precise antigen–adjuvant coupling enabled by genetic engineering and offers a roadmap toward intelligently designed next-generation vaccines.

A MOLECULAR TOOLKIT FOR PROGRAMMABLE IMMUNITY

Two principal technological pathways for synthesizing intramolecular adjuvants have become well-established. The chemically conjugated intramolecular adjuvants have been systematically reviewed before.⁸ Nevertheless, those constructed via genetic engineering strategies serve as well-defined molecular components. Through rational selection and combination, they can form a molecular toolkit for programmable immunity. By deconstructing their functions, we can classify them into distinct modules, each designed to execute a specific immunological task and targeted pathogens. This modular perspective transforms the vague notion of “adjuvanticity” into a series of defined, engineerable functions: targeting specific cells, activating defined signaling pathways, polarizing the immune response, and enhancing lympho-

cyte activation. Current research focuses primarily on four categories of intramolecular adjuvants: derivatives of bacterial toxins, PRR ligands, cytokines, and the complement fragments, each of which will be discussed in detail in the following sections (Table 1).

Bacterial toxin derivatives

Bacterial toxin-derived peptides represent an early class of intramolecular adjuvants. Through precise structural engineering, these molecules retain the receptor-binding capability of the parent toxin while being detoxified (Figure 2), and can be fused to antigens to enhance immunogenicity. For instance, intramolecular adjuvants derived from cholera toxin (CT) or *Escherichia coli* heat-labile enterotoxin (LT) retain the nontoxic B-subunit pentamer after removal of toxic domains.¹⁸ The C-terminal fragment of the tetanus toxin heavy chain (TTFC) maintains strong immunogenicity without neurotoxicity,¹⁹ and the diphtheria toxin derivative CRM197 incorporates a Gly-Glu point mutation that inactivates the toxic enzymatic domain.²⁰ Collectively, such modifications convert highly toxic native toxins into safe intramolecular adjuvants (Table 2).

Cholera toxin subunit. Cholera toxin B subunit (CTB), a nontoxic mucosal adjuvant, targets APCs by binding to ganglioside GM1, enhancing antigen uptake and eliciting antigen-specific mucosal and systemic antibody responses.²¹ The use of flexible linkers such as the GPMP hinge to connect CTB with antigens can help preserve the pentameric structure of CTB and the

Table 1. Intramolecular adjuvant classes in vaccine development: A comparative overview.

Adjuvant Class	Representative Molecule(s)	Targeted Receptor(s)	Immune Polarization	Core Advantage	Potential Limitation
Bacterial Toxins	CTB, LTB	GM1	Th1/Th2/ mucosal IgA	Potent mucosal delivery; breaches epithelial barriers	Potential off-target effects via GM1 binding; CIES
PRR Ligands	Flagellin	TLR5	Th1/Th2 (context-dependent); mucosal IgA	Bridges innate and adaptive immunity; induces balanced humoral and cellular responses	Full-length size restricts fusion design; eukaryotic glycosylation impairs TLR5 activity
	RS09	TLR4	Th1/Th2 (context-dependent)	Small peptide with simple structure; low inherent immunogenicity	Safety profile requires comprehensive <i>in vivo</i> validation
Cytokines	IFN α , IL2	IFNAR, IL2R	Th1/CTL	Directly modulates immune cell differentiation and response polarization	Risk of systemic toxicity and broken self-tolerance (autoimmunity)
Complement Fragments	C3d	CR2 (CD21)	Th2/humoral immunity	Lowers B-cell activation threshold; promotes affinity maturation	Marked species-specific differences in receptor-ligand interaction

Abbreviations: CTB, cholera toxin B subunit; LTB, heat-labile enterotoxin B subunit; CIES, carrier-induced epitope suppression; IFNAR, type I interferon receptor; IL2R, interleukin-2 receptor; PRR, pattern recognition receptor; TLR, Toll-like receptor; C3d, complement component 3d; CR2, complement receptor 2 (also known as CD21).

native conformation of the antigen, thereby maintaining GM1-binding ability and antigenic epitope presentation.²² Its high-affinity binding to GM1, which facilitates efficient antigen internalization through multivalent cross-linking and membrane curvature changes, governs membrane binding, endocytosis, and cellular targeting.²³ Nonetheless, GM1 binding also raises safety concerns, as it may disrupt normal cellular signaling and pose a hypothetical autoimmunity risk in susceptible populations.²¹ Notably, the full adjuvant effect of CTB extends beyond GM1 ligation and involves complex, indirect signaling pathways.

The intramolecular CTB fusion strategy has demonstrated adjuvant efficacy across bacterial, viral, and parasitic vaccine models, with divergent T helper polarization profiles that confirm its context-dependent immunomodulatory nature. In bacterial vaccines, fusion constructs including the ETEC adhesin chimera,²⁴ *Helicobacter pylori* multi-epitope fusion,²⁵ and *Streptococcus pneumoniae* PsaA fusion²⁶ consistently outperform physical antigen-CTB admixtures in antibody responses and protective efficacy via mucosal delivery, predominantly driving Th2-biased or mucosal antibody-enhanced responses. For viral vaccines, CTB fusion to the conserved H5N2 avian influenza stem antigen primarily augments humoral immunity, with elevated IgG titers and neutralizing activity. In a DNA prime-recombinant vaccinia boost heterologous regimen, by contrast, CTB fusion concurrently enhances antigen-specific T-cell responses in both systemic and multiple mucosal compartments, shifting toward a cell-mediated immune profile.²⁷ In parasitic vaccines, the *Eimeria tenella* CTB-MIC2 fusion induces concurrent elevation of Th1- and Th2-associated cytokines, with synchronously increased IFN γ and IL4 levels, raising oocyst reduction efficacy from 59.04% to 75.18% upon challenge.²⁸ Overall, CTB does not enforce a fixed single polarization phenotype, but modulates immune bias flexibly according to antigen properties, administration route and immunization regimen, establishing itself as a versatile adjuvant-carrier platform.

Beyond CTB, the cholera toxin subunits CTA1²⁹ and CTA2³⁰ can also serve as intramolecular adjuvants. In one study, CTA1 was directly fused with an anti-CD103 single-chain antibody and an ovalbumin epitope for dendritic cell (DC)-targeted melanoma therapy in mice. The ADP-ribosyltransferase activity of CTA1 enhanced antigen presentation by conventional type 1 DCs, promoting epitope cross-presentation and activation of CD8⁺ T cells and Th17 cells.²⁹

Heat-labile enterotoxin. Heat-labile enterotoxin B subunit (LTB), a non-toxic component of the LT produced by enterotoxigenic *Escherichia coli* (ETEC), shares approximately 80% amino acid sequence homology with CTB.³¹ Both belong to the bacterial AB5 toxin family and consist of an enzymatically active A subunit (LTA/CTA) noncovalently associated with a

pentameric ring of receptor-binding B subunits (LTB/CTB).³² Their ability to specifically bind the ganglioside GM1 receptor on eukaryotic membranes facilitates antigen internalization and DC activation, eliciting both mucosal and systemic immune responses, rendering them attractive intramolecular adjuvants.

The broad applicability of LTB has been demonstrated across multiple pathogen models. In mucosal vaccines, an LTB fusion with the PEDV COE epitope delivered orally via *Lactobacillus* induced high levels of mucosal sIgA and serum IgG, as well as a mixed Th1/Th2 response.³³ Similarly, fusion of LTB with multi-epitope antigens of *Mycoplasma hyopneumoniae* enhanced both local IgA and systemic IgG in pigs following intranasal or intramuscular immunization.³⁴ In aquaculture, a VP6-LTB fusion from grass carp reovirus delivered orally via *Lactococcus lactis* enhanced intestinal T cell activity and upregulated key type I interferon pathway genes, strengthening innate immunity.³⁵ A recombinant fusion of LTB with a *Clostridium perfringens* beta-toxin epitope offers a promising strategy for developing an effective and safe vaccine.³⁶ Owing to its well-characterized adjuvant properties and available robust expression systems, LTB has also been fused with the SARS-CoV-2 RBD antigen to improve mucosal immunization.³⁷

LTB has also been employed in adjuvant combination strategies. When used with CpG in a *Staphylococcus aureus*–*Streptococcus* multi-antigen vaccine (GIT), LTB synergistically enhanced both Th1 and Th2 responses, elevating IL2, IL4, IFN γ , and IL10 secretion, while significantly increasing IgG titers. This synergy likely stems from LTB's antigen-presentation enhancing effect combined with CpG-mediated TLR9 activation, resulting in amplified immune cascades.³⁸

Furthermore, the double-mutant heat-labile toxin (dmLT), a widely studied genetically engineered derivative of LT, carries point mutations such as R192G/L211A in its A subunit that substantially reduce enzymatic activity and toxicity while preserving GM1 ganglioside-binding capacity. This modification endows dmLT with excellent mucosal adjuvant activity and low enterotoxicity.³⁹ Notably, its single-mutant counterpart, LT (R192G), functions as an intramolecular adjuvant that potently enhances antibody responses against the poorly immunogenic heat-stable (STa) toxin, offering a novel molecular strategy for ETEC vaccine design.⁴⁰ Although dmLT is rendered non-toxic, the GM1-binding property necessitates rigorous exclusion of any cross-contamination with the active toxin form during production, and the balance between immunogenicity and reactogenicity must be carefully evaluated.

Tetanus toxoid T cell epitopes. Tetanus Toxoid (TT) is formaldehyde-inactivated tetanospasmin secreted by *Clostridium tetani*. The T cell epitopes derived from TT, rich in repetitive peptide sequences and amphipathic

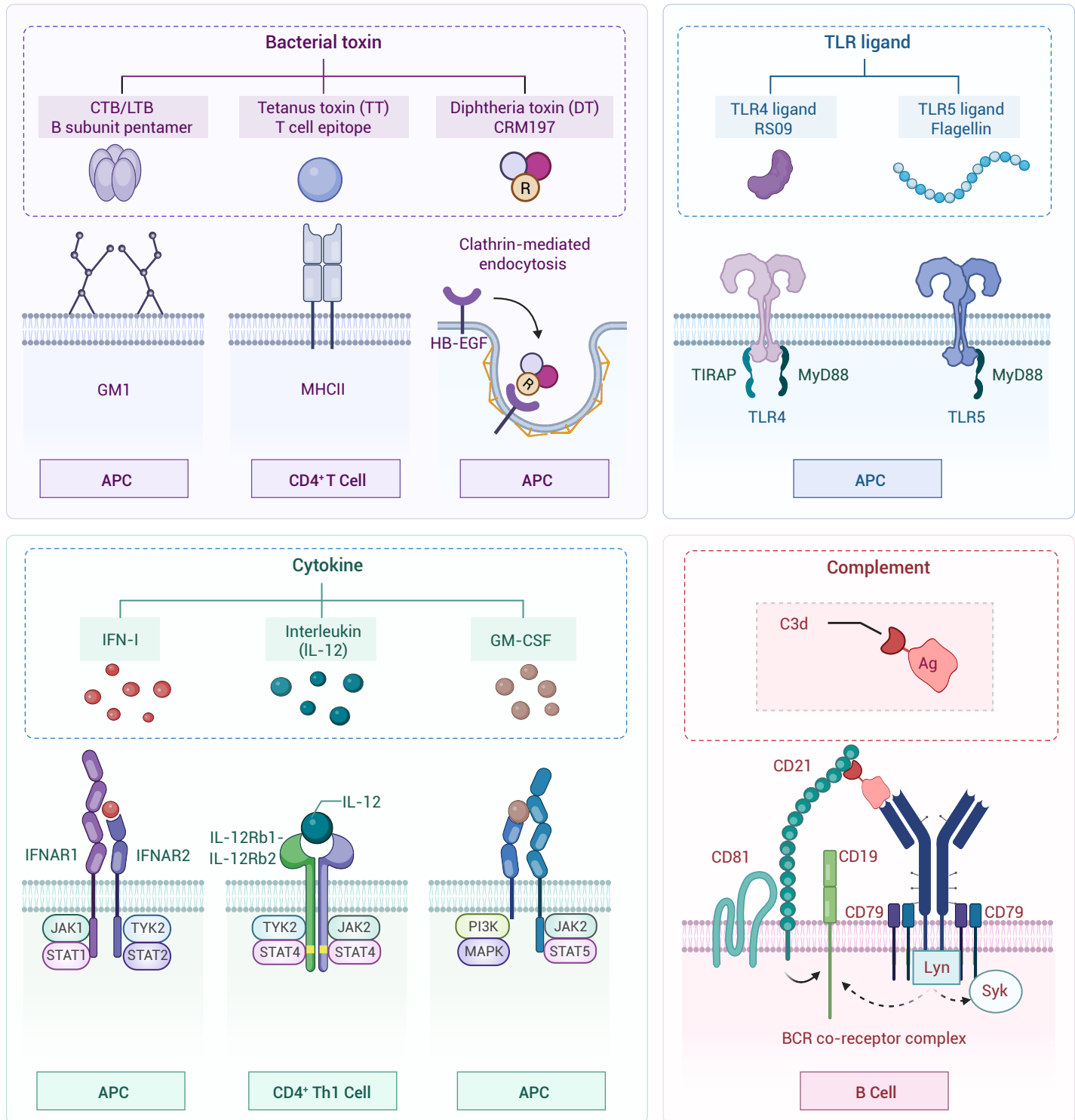


Figure 2. Mechanistic classification and signaling pathways of four major genetically engineered intramolecular adjuvants Genetically engineered intramolecular adjuvants are categorized into four functional classes: (1) bacterial toxin derivatives (e.g., CTB, LTB, CRM197, TT epitopes), (2) pattern recognition receptor ligands (e.g., flagellin for TLR5, RS09 for TLR4), (3) cytokines (e.g., IFN α , GM-CSF, IL12), and (4) complement fragments (primarily C3d). Each class operates through a distinct receptor-mediated mechanism depicted schematically. Bacterial toxin derivatives bind to cell-surface gangliosides (GM1) or HB-EGF, thereby targeting the fusion protein to APCs and facilitating internalization. PRR ligands directly engage toll-like receptors on the plasma membrane or endosomal compartments, activating MyD88/NF- κ B and IRF pathways to induce pro-inflammatory cytokines and co-stimulatory molecule expression. Cytokine adjuvants bind to their cognate receptors (e.g., IFNAR, GM-CSFR) to drive JAK-STAT signaling, directly polarizing Th1/Th2 differentiation and enhancing APC maturation and cross-presentation. Complement C3d fragments tether the fusion protein to CR2 (CD21) on B cells, co-clustering the B cell receptor with CD21 to lower the activation threshold and amplify both B-cell activation and antigen retention on follicular dendritic cells.

domains, can broadly bind to MHCII, functioning as universal T-helper epitopes to enhance antigen immunogenicity. Activation of CD4⁺ T cells by these TT epitopes promotes B cell differentiation and antibody affinity maturation, alongside the induction of a mixed Th1/Th2 immune response. Its advantage as an intramolecular adjuvant stems from the pre-existing

immunological memory resulting from widespread Diphtheria Tetanus Pertussis vaccination.⁴¹ This memory enables the enhancement of foreign antigen immunogenicity through T and B cell collaboration while avoiding epitope suppression. Nevertheless, limitations include possible interference from pre-existing antibodies and inter-individual variability in responses,

Table 2. Bacterial toxin derivatives as genetically engineered intramolecular adjuvants.

Intramolecular Adjuvant	Target Pathogen/Disease	Antigen	Fusion Construct Name(s)	Vaccine Delivery Platform	Adjuvant Mechanism	Experimental Model/Species	Ref .	
Cholera Toxin		<i>Eimeria tenella</i>	Microneme protein 2, (MIC2)	CTB-MIC2	Recombinant Subunit	GM1-mediated APC targeting	BALB/c mice; <i>E. tenella</i> -challenged chickens	28
	Cholera Toxin B subunit (CTB)	Alzheimer's Disease	Amyloid-β peptide (Aβ42)	CTB-Aβ42	Recombinant Subunit	GM1-mediated APC targeting	B6C3-Tg (<i>APP^{swe}</i> , <i>PSEN1^{dE9}</i>) transgenic mice	22
		Not specified (Immunomodulation study)	OVA	pSV-OVA-CTB, rTTV-OVA-CTB	DNA vaccine; Recombinant Tiantan vaccinia	GM1-mediated APC targeting	BALB/c mice	27
	Cholera Toxin A1 subunit (CTA1)	Murine melanoma	OVA epitopes (257-264, 323-339)	CTA1-I/II-aCD103	Recombinant Subunit	Enhanced ADP-ribosylation activity	C57BL/6J mice; OT-I transgenic mice	29
Heat-Labile Enterotoxin B subunit (LTB)		<i>Clostridium perfringens</i>	Beta toxin epitope (btx140-156)	rLTB-Btx140-156	Recombinant Subunit	GM1-mediated APC targeting	<i>In silico</i> ; <i>In vitro</i>	36
		Porcine Epidemic Diarrhea Virus	Core Neutralizing Epitope (COE)	LTB-COE	Recombinant <i>L. casei</i>	Enhanced mucosal immunity	BALB/c mice	33
		<i>S. aureus</i> / <i>S. dysgalactiae</i>	GIT fusion protein	LTB-GIT	Recombinant Subunit	Enhanced Th1/Th2 responses via GM1 binding	BALB/c mice	38
		Grass Carp Reovirus (GCRV-II)	VP6 protein	VP6-LTB	Recombinant <i>L. lactis</i>	Boosted mucosal and systemic immunity	Rare minnow	35
		<i>Mycoplasma hyopneumoniae</i>	P97 adhesin R1 repeat region	rLTB-R1-P42-NrdF	Recombinant Subunit	Boosted mucosal and systemic immunity	BALB/c mice; Swine	34
		SARS-CoV-2 (COVID-19)	Receptor-Binding Domain (RBD)	LTB-RBD	Recombinant Subunit	Enhanced mucosal immunity & antigen presentation	BALB/c mice	37
Tetanus Toxin P2 (TT830-844) (TT)	Rotavirus	Truncated VP8* protein	P2-P[8]ΔVP8*	Recombinant Subunit	Boosted neutralizing antibodies	Guinea pigs; Gnotobiotic piglets	44	
	Foot-and-Mouth Disease Virus (FMDV)	ΔVP1 and 2×B1B2 epitopes	FT, FME, FTME	Recombinant Subunit	Enhanced T-cell immune response	BALB/c mice; Guinea pigs	45	
Cross-reactive Material 197 (CRM197)	Colon Carcinoma	Basic Fibroblast Growth Factor (bFGF)	CRM197-bFGF (CF)	Recombinant Subunit	Boosted mixed Th1/Th2 responses	BALB/c mice	52	
	Hepatitis E Virus (HEV)	ORF2 (aa 394-606, E2)	CRM197(A)-E2	Recombinant Subunit	Boosted mixed Th1/Th2 responses	BALB/c mice; Cynomolgus monkeys	53	

Abbreviations: APC, antigen-presenting cell; GM1, monosialotetrahexosylganglioside; Th, T helper cell; GIT, a fusion protein antigen (from *S. aureus* and *S. dysgalactiae*); VP6, viral protein 6; Δ VP1, deleted VP1 protein; B1B2, tandem B cell epitopes; ORF2/E2, open reading frame 2 protein/E2 domain. Species names are italicized in the 'Target Pathogen/Disease' column.

attributable to MHC polymorphism. More recently, defined TT segments such as the P2 (aa 830-844)⁴² and P30 (aa 947-967)⁴³ epitopes have been used in vaccine design, where they are linked to target antigens via genetic fusion or chemical conjugation to improve vaccine breadth and efficacy.

In a rotavirus vaccine design, genetic fusion of the P2 epitope to the N-terminus of Δ VP8* enhanced serum neutralizing-antibody and VP4-specific IgG responses. In gnotobiotic piglets, it delayed diarrhea onset and reduced disease duration and cumulative diarrhea score.⁴⁴ A similar approach has been explored for foot-and-mouth disease virus (FMDV) vaccines. A flagellin-TT-P2 fusion protein was shown to activate the TLR5/NF- κ B pathway, promoting the secretion of cytokines like TNF α and IL12 and inducing a Th1-polarized response. Consequently, it elicited potent neutralizing antibody responses against the homologous challenge strain.⁴⁵

Conversely, the TT-P30 epitope has been successfully utilized via chemical conjugation in a vaccine targeting the tumor-associated antigen MUC1 glycopeptide. P30 possesses three independent MHCI-binding motifs. When conjugated to MUC1 glycopeptides, it induced robust high-titer specific IgG antibodies without Freund's adjuvant, demonstrating superior immunostimulatory effects compared to conventional P2 and P4 epitopes. Mechanistic studies indicate that the P30 epitope activates both Th2- and Th1-type immune responses, leading to effective tumor cell clearance via complement-dependent cytotoxicity and enabling synergistic humoral and cellular immunity, making it a promising candidate for rational anticancer vaccine design.⁴³

The core principle of applying TT-derived T cell epitopes lies in "hijacking" pre-existing memory CD4⁺ T cells elicited by prior immunization. These memory T cells provide rapid and potent helper signals to the linked neoanti-

gen, accelerating the initiation of immune responses. Nevertheless, the application of this strategy is challenged by MHC polymorphism, which drives inter-individual variation in response magnitude, and by the pre-programmed polarization of the pre-existing memory response, which can bias the immune response towards the neoantigen, thereby limiting the vaccine's broad applicability and predictive accuracy. Therefore, its application requires comprehensive consideration of population immune backgrounds, antigen properties, and the desired response type to rationally optimize vaccine design.

Cross-Reactive Material 197 (CRM197). CRM197 is a nontoxic mutant of diphtheria toxin (DT), generated by a single amino acid substitution (G52E).²⁰ This mutation inactivates the catalytic domain (fragment A) of DT, abolishing enzymatic activity and toxicity, but spares the receptor-binding domain (R domain, aa 387–535) and the overall immunogenicity of the DT protein. The R domain enables specific binding to the cell-surface heparin-binding epidermal growth factor receptor (HB-EGF), triggering receptor-mediated endocytosis.⁴⁶ Beyond its safety profile, CRM197 exhibits high structural plasticity, allowing flexible conjugation to diverse targets such as polysaccharides, tumor antigens, and viral epitopes.^{47–51} Antigen complexes formed via genetic fusion or chemical conjugation are internalized by DCs or macrophages. Subsequent peptide presentation via MHCII activates CD4⁺ T cells, effectively converting T-independent antigens into T-dependent antigens and promoting long-lasting humoral and cellular immunity. Notably, genetic fusion strategies can avoid batch variability associated with chemical conjugation, highlighting the promising vaccine potential of CRM197.

In cancer immunotherapy, basic fibroblast growth factor (bFGF), a pro-angiogenic factor, has been targeted despite its weak inherent immunogenicity. A fusion protein of bFGF with CRM197, combined with CpG/alum adjuvants, induced a mixed Th1/Th2 response and high-titer specific antibodies, significantly suppressing angiogenesis and promoting tumor cell apoptosis in a mouse model of colon cancer.⁵² This antitumor effect was closely associated with CRM197-mediated activation of antigen-specific B cells and modulation of cytokine secretion. CRM197 also offers advantages in viral vaccine design beyond conventional particulate antigens. For instance, fusion of the E2 protein (aa 394–606) of hepatitis E virus (HEV) with the catalytic domain (fragment A) of CRM197 yielded a dimeric CRM197(A)-E2 protein that markedly enhanced the immunogenicity of the non-particulate antigen, raising antibody titers by 10- to 1000-fold compared with the particulate antigen p239.⁵³

A key limitation, however, relates to certain levels of pre-existing anti-CRM197 immunity, as this protein has long served as the carrier component in multiple licensed pediatric conjugate vaccines.⁵⁴ High levels of anti-CRM197 antibodies in individuals can lead to carrier-induced epitope suppression (CIES),⁵⁵ where a strong immune response against the familiar carrier protein suppresses responses to the conjugated neo-antigen. This is an important consideration in the clinical deployment of CRM197 as a protein carrier.

Tandem arrangement of cytotoxic T lymphocyte (CTL) and helper T cell (Th) epitopes, combined with terminal adjuvant fusion, represents a core strategy to improve the immunogenicity of multi-epitope vaccines. Relevant studies have validated this design in multiple pathogen vaccine developments. For instance, fusion of hepatitis C virus non-structural protein-derived CTL epitopes with PADRE Th epitope, *meningococcal* lipopeptide and IL2 adjuvants can promote DC maturation and trigger robust Th1 and Th2 immune activation.⁵⁶ Similarly, a *Mycobacterium tuberculosis* multi-epitope vaccine constructed by tandem linkage of multiple HTL and CTL epitopes with TLR2 agonists and PADRE sequences elicited strong antigen-specific antibody and IFN γ responses in mice, lowered bacterial burden, and blocked pathogen extrapulmonary dissemination.⁵⁷ Functional linker peptides including AAY, GPGPG and EAAAK are widely applied in vaccine design to isolate distinct epitopes and preserve their biological activities. Specifically, the rigid EAAAK linker eliminates steric hindrance between adjuvant and epitope domains, the flexible GPGPG linker optimizes MHC II-mediated antigen presentation of Th epitopes, and the AAY linker facilitates precise proteasomal cleavage and CTL epitope release. Beyond pathogen-specific epitopes, synthetic universal Th epitopes have overcome pathogen and HLA restriction, emerging as prevalent intramolecular adjuvant components. The classic

PADRE epitope has been applied in a cytomegalovirus pp65-based vaccine candidate, which has completed Phase I clinical evaluation with satisfactory safety.⁵⁸ Other synthetic universal Th epitopes such as TpD, a chimeric peptide that integrates tetanus toxoid and diphtheria toxoid epitopes with an internal cathepsin cleavage site, has also been shown to induce robust CD4⁺ memory T cell recall responses in mice, non-human primates, and humans.⁵⁹

Bacterial toxin derivatives including CTB/LTB and tetanus toxoid epitopes are well suited for mucosal pathogens and low-immunogenic antigens that rely on robust CD4⁺ T cell support. CTB/LTB fusion constructs induce strong mucosal IgA and systemic IgG responses, making them promising candidates for diarrheal and respiratory mucosal vaccines. TT epitopes and CRM197 strengthen immune responses by utilizing pre-existing T-cell memory and enabling T-dependent immunity for poorly immunogenic antigens.

Pattern recognition receptor (PRR) ligands

Pattern recognition receptors (PRRs), the core recognition components of the innate immune system, are pivotal to vaccine adjuvant development due to their recognition of pathogen- and damage-associated molecular patterns (PAMPs/DAMPs).⁶⁰ Their specific agonists, such as ligands for Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and the stimulator of interferon genes (STING), can be co-expressed or fused with antigens to activate PRR signaling in APCs. This activation upregulates costimulatory molecules, promotes the secretion of proinflammatory cytokines including IL6 and TNF α , and subsequently modulates Th1/Th2 balance while inducing antigen-specific CTL responses, representing a breakthrough direction in novel vaccine design (Table 3). Notably, signaling through most PRRs, including TLRs, can induce localized tissue and cellular damage, while the DAMPs released thereby appear to be a critical component of the adjuvant activity of several vaccine adjuvants.⁶¹

The TLR family, the most extensively studied subset of PRRs, comprises 10 members (TLR1–TLR10) that exhibit marked ligand specificity: TLR2 recognizes bacterial lipoproteins (e.g., macrophage-activating lipopeptide-2, MALP-2), TLR4 binds to Gram-negative bacterial lipopolysaccharide (LPS) and endogenous heat shock proteins (HSPs), TLR5 detects the conserved domain of flagellin (Figure 2), and TLR9 specifically responds to unmethylated CpG DNA motifs. Since the identification of the first human Toll-like receptor (TLR4) in 1997,⁶² understanding of TLR function has deepened considerably, revealing that TLR signaling not only contributes to pathogen clearance but also exerts dual roles in processes such as tumor immune evasion, autoimmune diseases, and metabolic syndrome, thereby serving as a critical molecular bridge linking innate and adaptive immunity.⁶³ TLR agonists have now demonstrated substantial potential as vaccine adjuvants.^{64,65} A wide range of intramolecular adjuvants based on PRR ligands, including those targeting TLRs, NLRs, CLRs, and RLRs, often chemically conjugated, have been extensively studied.⁸ In particular, ligands for TLR2, TLR4, TLR7/8, and TLR9 can activate immune responses via distinct mechanisms. This review focuses on intramolecular adjuvant strategies employing PRR ligands genetically fused to antigens using genetic engineering technology.

TLR4-targeting intramolecular adjuvant: RS09. TLR4 ligands are highly diverse, ranging from LPS to HSPs. RS09 (APPHALS) is a synthetic heptapeptide rationally designed to functionally mimic lipid A and trigger TLR4 signaling. Unlike modified LPS derivatives such as MPL, it is not chemically processed from natural lipid A, and it lacks the toxic structural motifs carried by native lipopolysaccharide. As a TLR4 agonist, RS09 promotes NF- κ B nuclear translocation and induces inflammatory cytokine secretion in macrophages, making it a promising intramolecular adjuvant in novel vaccine design.⁶⁶ To date, several immunoinformatics studies have incorporated RS09 into multi-epitope vaccine constructs against malaria,⁶⁷ Chikungunya virus,⁶⁸ Schistosoma,⁶⁹ and Ascaris,⁷⁰ in which molecular docking and dynamics simulations predicted stable binding of the RS09-fused constructs to TLR4, and immune simulations suggested enhanced antibody and T cell responses. However, systematic *in vivo* validation of these multi-epitope RS09 fusion proteins remains lacking. Beyond computational design, RS09 has been evaluated as a genetically encoded adjuvant in an adeno-associated virus (AAV)-vectored SARS-CoV-2 vaccine, where an RS09-fused RBD

Table 3. TLR ligands as genetically engineered intramolecular adjuvants.

Intramolecular Adjuvant	Target Pathogen/Disease	Antigen	Fusion Construct Name(s)	Vaccine Delivery Platform	Adjuvant Mechanism	Experimental Model/Species	Ref.
TLR4 agonist: RS09	SARS-CoV-2 (COVID-19)	RBD (aa 330-583)	S663V-RBD	Recombinant AAV-vectored vaccine	Enhanced Th1/Th2 responses via TLR4 activation	BALB/c mice; C57BL/6 mice	71
	<i>Plasmodium falciparum</i> (Malaria)	Multi-epitope construct	Multi-epitope-RS09	Recombinant Subunit	Enhanced TLR4-mediated immunity	<i>In silico</i>	67
	<i>Streptococcus pneumoniae</i> / Aging-related immunological defects	Pneumococcal surface protein A (PspA)	FlaB-PspA (FP)	Recombinant Subunit	Activation of TLR5 pathway	C57BL/6J mice; TLR5 knockout mice	72
	Foot-and-Mouth Disease Virus (FMDV)	Δ VP1 and 2x B1B2 epitopes	FT, FME, FTME	Recombinant Subunit	Activation of TLR5 pathway	BALB/c mice; Guinea pigs	45
TLR5 agonist: Flagellin	Human Papillomavirus (HPV)	RG1 epitope of L2 protein	d3RG-T5 (Flagellin D1)-TT-T4 (RS09)	Recombinant Subunit	TLR5 activation enhancing Th2 immunity	BALB/c mice	66
	Vaccinia Virus	L1R & B5R proteins	L1R-Flagellin (LF)	Recombinant Subunit	TLR5 activation enhancing Th2/complement immunity	BALB/c mice	73
	Porcine Epidemic Diarrhea Virus (PEDV)	PEDV spike protein truncated region S0	S0-F (C-terminal fusion), F-S0 (N-terminal fusion)	Recombinant Subunit	Enhanced humoral immunity	BALB/c mice	74

Abbreviations: AAV, adeno-associated virus; L2-protein, the minor capsid protein L2 of Human Papillomavirus; RBD, receptor-binding domain (of the SARS-CoV-2 spike protein); RG1 epitope, a conserved, immunodominant epitope within the HPV L2 protein capable of inducing broadly neutralizing antibodies; S0, a defined, truncated region of the PEDV spike protein; Δ VP1, a truncated or internally deleted form of the VP1 structural protein of FMDV.

Bacterial genus and species names are italicized in the 'Target Pathogen/Disease' column.

domain was packaged into an AAV vector. Following intramuscular injection, host cells transcribed and translated the RS09-RBD fusion protein, which self-assembled into a trimeric conformation mimicking the native viral spike. This single-dose vaccine elicited rapid, robust, and long-lasting RBD-specific antibody responses and conferred superior neutralizing activity against multiple SARS-CoV-2 pseudovirus variants in mice.⁷¹ Collectively, these results confirm the *in vivo* intramolecular adjuvant activity of RS09 delivered via gene vector platforms, while its protective efficacy following direct administration as a recombinant fusion protein remains unclarified.

TLR5-targeting intramolecular adjuvant: Flagellin. Flagellin is recognized by TLR5, activating downstream NF- κ B signaling pathways and inducing the release of innate immune effectors such as proinflammatory cytokines, type I interferons (IFNs), and nitric oxide, thereby significantly enhancing both humoral and cellular immune responses. Its unique four-domain architecture (conserved D0/D1 and hypervariable D2/D3 regions) confers flexible adjuvant activity. When expressed as a fusion with an exogenous antigen, flagellin synergistically activates DCs and promotes cytokine-mediated migration to lymphoid organs, efficiently bridging innate and adaptive immunity. A study developed a nasally delivered mucosal vaccine by fusing the pneumococcal antigen PspA with flagellin (FlaB), demonstrating dual efficacy in combating infections and mitigating aging-related decline. Mechanistic investigations revealed that FlaB-mediated TLR5/NF- κ B pathway activation enhances IL22 and secretory IgA production while concurrently suppressing pro-inflammatory senescence-associated signals.⁷² In an FMDV vaccine study, three different flagellin-antigen epitope fusion proteins were designed, which significantly improved antibody titers and protective efficacy.⁴⁵ Other studies have also confirmed that flagellin can enhance adaptive immune responses to weakly immunogenic proteins.^{73,74} Besides, in adenovirus vaccine studies, two *Salmonella* flagellin proteins (FljB and FljC) have been used as adjuvants. In mice, co-expression of FljB with the rabies virus glycoprotein (RVG) in an HA5 vector stimulated a stronger Th2-biased

antibody response than the FljC-containing vaccine.⁷⁵ This difference in adjuvant effect may relate to overall molecular features of flagellin subtypes, though the precise structural mechanisms require further investigation. On the other hand, FlaB expressed in eukaryotic systems shows greatly reduced ability to activate TLR5 signaling due to glycosylation modifications, which is crucial for the design of flagellin-based molecular adjuvant vaccines.⁷⁶ Furthermore, the large size of full-length flagellin poses challenges for fusion construct design. Efforts to identify minimal functional regions have revealed that both the D0 and D1 domains are required for TLR5 activation, as the D1 domain mediates primary binding to TLR5 while the D0 domain, particularly its C-terminal segment, is essential for stabilizing the 2:2 signaling complex. In the absence of D0, TLR5 dimerization and downstream signaling are abrogated. These structural constraints underscore the need for rational truncation strategies that preserve both domains to maintain adjuvant potency.^{77,78}

Multimodal receptor co-activation can overcome the immune bias associated with single signaling pathways. In an HPV vaccine fusion protein system, a dual TLR4/5 agonist (RS09 + flagellin D1 domain) synergistically activated the MyD88-NF- κ B pathway, significantly elevating neutralizing antibody levels, while this effect was further enhanced by the addition of the exogenous TLR7 agonist R837, optimizing Th1/Th2 balance. Molecular docking studies indicated that the two adjuvant molecules bind distinct sites on TLR4/MD2 and TLR5, respectively, triggering complementary signaling cascades.⁶⁶ This design overcomes limitations of single-adjuvant approaches, demonstrating that combining multiple intramolecular adjuvants with external adjuvants can substantially improve vaccine efficacy. However, this potent combinatorial activation of multiple TLR pathways warrants careful safety consideration. TLR4 is the cognate receptor for bacterial lipopolysaccharide, and studies have shown that TLR4 overexpression can exacerbate lipopolysaccharide-induced inflammatory responses and apoptosis, as well as lead to autophagic dysfunction and tissue damage via the NF- κ B/MAPK signaling pathway.⁷⁹ Similarly, high-dose flagellin has

been shown to induce acute liver injury through over-activation of TLR5 signaling, characterized by hepatocellular necrosis, oxidative stress, and neutrophil infiltration.⁸⁰ As RS09 and flagellin both signal through the MyD88–NF- κ B axis, their combined use may synergistically amplify downstream inflammatory cytokine cascades, raising concerns about potential synergistic toxicity.

It is noteworthy that the current genetically encoded intramolecular adjuvants targeting TLRs are predominantly protein-based agonists of surface-expressed receptors, such as flagellin for TLR5 and RS09 for TLR4. This reflects a fundamental constraint imposed by the chemical nature of PRR ligands: proteinaceous ligands can be seamlessly integrated into the genetic code as fusion partners, whereas many potent agonists of endosomal TLRs are non-proteinaceous molecules. For instance, the ligands for TLR7 and TLR8 are typically small organic compounds such as imidazoquinolines, and the ligands for TLR9 are CpG oligodeoxynucleotides.⁸¹ These molecules cannot be genetically encoded and must be attached to antigens via chemical conjugation strategies. The choice between genetic fusion and chemical conjugation is therefore dictated not merely by technical preference but by the biosynthetic nature of the ligand itself, a consideration that has important implications for expanding the repertoire of intramolecular adjuvants to include endosomal PRR pathways.

PRR ligands instruct innate immunity to elicit humoral and cellular immune responses, with a context-dependent Th1/Th2 profile shaped by the ligand, antigen, and delivery route. Flagellin confers balanced mucosal immune responses and is applicable to mucosal extracellular bacterial infections.⁷² Combined ligand administration integrates diverse signaling cascades to compensate for the deficiency of single agonists, enabling effective vaccination against sophisticated pathogens and challenging targets including malaria.

Cytokine-based intramolecular adjuvants

Cytokines act as potent immunostimulants by enhancing the immunoregulatory functions of natural killer cells, promoting T lymphocyte differentiation, broadly upregulating immune responses, and protecting the host against bacterial, viral, and parasitic challenges. By incorporating the genetic code for a specific cytokine into a vaccine construct, either as a direct fusion to the antigen or for co-expression from the same vector, it is possible to create a high local concentration of that immunomodulatory signal precisely at the site of antigen presentation. This strategy allows for direct and potent control over the polarization of the ensuing immune response. However, natural cytokines often suffer from short half-lives and limited stability *in vivo* due to susceptibility to proteolysis, pH variations, and rapid renal clearance.^{82,83} The intramolecular adjuvant approach, which fuses cytokines with antigenic proteins, significantly prolongs their persistence *in vivo* and optimizes the spatiotemporal coordination of immune responses. Cytokines such as interferons, interleukins, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have demonstrated considerable adjuvant potential in novel vaccine designs, particularly nucleic acid-based vaccines, representing an important direction in molecular immunology engineering (Table 4).^{84–86} While cytokine-encoded nucleic acid vaccines have been previously reviewed,⁸⁵ we particularly emphasize the intramolecular adjuvant form, that is, the form where the cytokine is fused with the antigen.

Interferon family. The interferons (IFNs) family enhances antiviral and antitumor immunity by initiating the JAK–STAT signaling pathway and inducing interferon-stimulated genes (ISGs). IFN α is regarded as a natural immune adjuvant that promotes DC maturation and enhances Th1-type cytokine secretion, thereby strengthening both humoral and cellular immune responses. In the approved fusion protein vaccine V-01, the IFN α -PADRE-RBD-Fc dimer design induced durable neutralizing antibody production and a Th1-polarized T cell response in clinical trials, with the Fc domain significantly extending the protein's half-life and improving its pharmacokinetic profile.^{11,87} A similar design has also been applied to the herpes zoster gE protein.⁸⁸ For IFN β , fusion with albumin (Alb-IFN β) increased its half-life to 48 hours and leveraged albumin's lymph-targeting properties to promote DC secretion of CXCL9/CXCL10, markedly enhancing HPV E7 antigen-specific CD8⁺ T cell expansion and tumor clearance.⁸⁴ Although IFN γ exhibits relatively low intrinsic immunogenicity, its fusion with HIV gp120 via a 34-aa flex-

ible linker (pAM-16) retained 88% of bioactivity and induced a stronger Th1 response compared to physically mixed formulations, underscoring the advantage of intramolecular fusion in preserving cytokine function.⁸⁹

Interleukin family. Multiple interleukins, including IL2,⁹⁰ IL4,⁹¹ and IL12,^{92,93} are considered to possess vaccine adjuvant potential and can provide strong immune co-stimulatory signals. As key regulators of the immune system, they activate diverse immune cells such as T cells, NK cells, and DCs, enhancing their proliferation and secretion of effector molecules such as IFN γ and TNF α , thereby amplifying antigen-specific immune responses. Moreover, T follicular helper cells are indispensable for high-quality sustained antibody responses by mediating germinal center reactions, making Tfh-targeted intramolecular adjuvants a promising route to optimize humoral immunity. Strategies such as nucleic acid vaccine design, delivery system optimization, and Fc⁹⁴ or albumin fusion have been employed to prolong their *in vivo* activity and precisely direct immune polarization. For example, IL12 drives Th1 immune polarization via STAT4 signaling and has shown strong adjuvant activity in novel nucleic acid vaccines. A recent study developed a pVAX1-PSA-IL12 fusion DNA vaccine, which significantly increased PSA-specific antibody levels and the frequency of IFN γ -secreting antigen-specific T cells, inhibiting tumour growth and prolonging survival in a mouse prostate cancer model.⁹⁵ Advances in mRNA-LNP technology have enabled precise delivery of IL2: the nucleoside-modified, albumin-fused IL2 mRNA nanoformulation (Alb-IL2 RNA-NP) maintained prolonged cytokine availability in tumor-bearing mice for up to 48 hours and, when combined with an RNA-LPX vaccine and PD-L1 blockade, markedly expanded antigen-specific CD8⁺ T cells and significantly improved survival in a subcutaneous mouse tumor model.⁹⁶ IL4 promotes Th2 polarization and IgG1 class switching via the STAT6 pathway. Co-expression of IL4 with the botulinum toxin AHC antigen increased neutralizing antibody titers by 4–8-fold and boosted survival to 100%.⁹⁷

Granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF directly promotes DC maturation and antigen cross-presentation, and its adjuvant activity has been confirmed in multiple studies.⁸⁶ However, the molecular design strategy profoundly influences immunogenicity and safety: co-expression and fusion expression lead to fundamentally different immunological outcomes. In a DNA prime–adenovirus boost immunization model, bicistronic vector-mediated co-expression of GM-CSF and ovalbumin (OVA) antigen significantly increased the proportion of CD8⁺ T cells. By contrast, fusing GM-CSF to OVA via a flexible (G4S)₃ linker to form a single protein (GM-OVA) led to high-titer neutralizing antibodies against GM-CSF due to epitope exposure, which subsequently suppressed CD8⁺ T cell responses and raised autoimmunity concerns.⁹⁸ These findings underscore the importance of spatiotemporal distribution and conformational integrity of cytokines in the local microenvironment: co-expression preserves the native structure and autonomous function of GM-CSF, whereas fusion may disrupt immune tolerance and cause adverse effects. In a tuberculosis vaccine study, co-expression of *Mycobacterium tuberculosis* Hsp65–Esat6 antigen with GM-CSF markedly enhanced CD4⁺/CD8⁺ T cell proliferation and IFN γ secretion without inducing autoimmunity.⁹⁹ Similarly, in a DNA cancer vaccine targeting carcinoembryonic antigen (CEA), a GM-CSF-CEA fusion protein elicited a Th1 response, but its antitumor efficacy was diminished by the generation of anti-GM-CSF neutralizing antibodies.¹⁰⁰ Furthermore, GM-CSF can be combined with other intramolecular adjuvants to synergistically modulate immune responses.³⁰

In summary, cytokine adjuvants enable rational immune programming tailored to pathogen clearance mechanisms. By leveraging strategies such as co-expression, genetic fusion, chemical conjugation, or delivery system integration, they precisely modulate the direction of immune responses. Type I interferons and IL12 drive Th1 and CTL responses, making them the adjuvants of choice for intracellular pathogens and therapeutic cancer vaccines, while IL4 promotes Th2 immunity ideally suited for neutralizing bacterial exotoxins and extracellular threats. Their core advantage lies in the ability to precisely regulate immunity through the endogenous signaling language of the immune system. However, the physical linkage of exogenous antigens to self-cytokines carries the risk of breaking immune tolerance. When endogenous molecules like GM-CSF are fused to vaccine antigens, potent T cell help directed against the foreign antigen may non-specifically activate B cells recognizing self-cytokine epitopes, potentially triggering autoimmunity.

Table 4. Cytokines as genetically engineered intramolecular adjuvants.

Intramolecular Adjuvant	Target Pathogen/Disease	Antigen	Fusion Construct Name(s)	Vaccine Delivery Platform	Adjuvant Mechanism	Experimental Model/Species	Ref.	
Interferons (IFNs)	IFN α	SARS-CoV-2 (COVID-19)	RBD (aa 319-541)	IFN α -RBD-Fc (I-R-F)	Recombinant Subunit	IFN α activates dendritic cells to enhance Th1/CTL responses	BALB/c mice; C57BL/6J mice; Rhesus macaques; Human clinical trial (Phase I/II)	11
	IFN β	Human Papillomavirus (HPV)	OVA, HPV E7	Albumin-IFN β (Alb-IFN β)	Recombinant Subunit	Extended half-life, targeted lymph node delivery	C57BL/6 mice; Batf3 knockout mice	84
	IL12	Prostate Cancer	Prostate-Specific Antigen (PSA)	pVAX1-PSA-IL12	DNA vaccine	Boosted Th1/CTL responses	C57BL/6 mice; RM-1 murine prostate cancer cell line	95
Interleukins	IL2	Colorectal Cancer/Melanoma	OVA, gp70, TRP-1	Alb-IL2 RNA-NP	mRNA-LNP vaccine	Extended half-life, targeted tumor delivery	C57BL/6J mice; BALB/cJRj mice; Murine tumor cell lines	96
	IL4	Botulinum Neurotoxin A	Hc domain	pSCARSAHc-IRES-IL4	DNA vaccine	Enhanced Th2 humoral response	BALB/c mice; C57BL/6 mice	97
Granulocyte-macrophage Colony-stimulating Factor (GM-CSF)		OVA model antigen	Ovalbumin (OVA)	GM-CSF-OVA	DNA vaccine	Induction of autoantibodies	C57BL/6N mice; OT-I transgenic mouse splenocytes	98
		<i>Mycobacterium tuberculosis</i>	Hsp65-Esat-6 fusion protein	pIRES-Hsp65-ESAT-6-GM-CSF	DNA vaccine	Boosted Th1 & Th2 responses	C57BL/6 mice	99
			OVA epitope (SIINFELK)	mG-CTA2/(CTB-OVAT) ₅	Recombinant Subunit	DC activation enhancing CD8 ⁺ T cell responses	C57BL/6J mice	30
		Prostate Cancer						

Abbreviations: CTL, cytotoxic T lymphocyte; Hc, heavy chain domain; mRNA-LNP, lipid nanoparticle-formulated messenger RNA; pIRES, internal ribosome entry site-containing plasmid vector; pSCARSAHc, plasmid vector for Clostridium antigen expression; pVAX1, mammalian expression plasmid. Bacterial genus and species names are italicized in the 'Target Pathogen/Disease' column.

Therefore, the decision between fusion and co-expression strategies represents a fundamental balance between adjuvant potency and safety. Further exploration is required to elucidate how endogenous pathways can be harnessed effectively while minimizing the risk of autoimmunity. Beyond the cytokines discussed above, numerous others, such as IL9,¹⁰¹⁻¹⁰³ IL17,^{104,105} and IL21,^{106,107} also possess intramolecular adjuvant potential. As research into their signaling pathways and immunomodulatory mechanisms deepens, it may become possible to design and "customize" more efficient engineered adjuvants tailored to specific immune response needs, thereby expanding the design possibilities for molecular vaccines.

Complement fragments-derived intramolecular adjuvants

The complement system is a key component of innate immunity, contributing to host defense through opsonization, release of inflammatory mediators, and formation of the membrane attack complex.¹⁰⁸ Among its components, C3d, the terminal cleavage fragment of complement C3, has gained attention as an intramolecular adjuvant due to its marked immunomodulatory functions (Table 5). Generated via the sequential degradation of C3b, C3d binds specifically to complement receptor 2 (CR2/CD21) on B cells, lowering the B-cell activation threshold and promoting BCR–CR2 co-crosslinking to amplify B-cell activation signals (Figure 2).^{109,110} This dual-receptor engagement mechanism greatly enhances antibody affinity maturation and prolongs antigen retention on follicular dendritic cells (FDCs), thereby supporting immune memory formation.

In modern vaccine design, C3d fusion has been applied to mRNA platforms. An mRNA encoding a SARS-CoV-2 spike protein fused to C3d, delivered via lipid nanoparticles (LNPs), induced antibody titers 10-fold higher than the non-fused antigen and significantly improved neutralization against the Delta variant.¹¹¹ And fusing C3d with the HIV-1 Env trimer through a 60-aa flexible linker can enhance CR2 binding while maintaining the antigen's native

conformation, increasing germinal center B cell activation by 2.1 times and remarkably improving antibody affinity.¹¹² Recent studies have further refined the functional domain of C3d, employing the minimal CR2-binding P28 peptide¹¹³ and site-specific conjugation to reduce antigenic epitope masking.¹¹⁴ Notably, C3d's adjuvant activity depends critically on multivalent binding: a single copy may sterically hinder epitope exposure, whereas a triple-tandem repeat (C3d₃) forms multivalent CR2-binding clusters, increasing anti-hCG β antibody titers by 243-fold in mice and inducing a Th2-biased response.¹¹⁰ Efficient lymphocyte activation generally requires extensive receptor crosslinking and clustering. Genetic engineering offers unique advantages in this context, enabling precise control over adjuvant valency to systematically optimize this biological effect, a level of precision difficult to achieve with chemical conjugation.

Beyond C3d, other complement components such as a C3b fragment (aa 727–767) have been explored as potential adjuvants. For example, fusing C3b to a viral RBD domain via a flexible linker targets CR1 and elicits more durable antibody responses than an Fc-fusion approach, broadening the scope of complement-based molecular adjuvants.¹¹⁴ Additionally, C1q, the initiating recognition molecule of the classical pathway, can facilitate targeted antigen delivery by binding to the CD91 receptor on APCs via its collagen-like tail. Purified C1q protein chemically conjugated to HIV virus-like particles has been shown to markedly enhance both mucosal and systemic immune responses following sublingual immunization.¹¹⁵ Similarly, C5a, a common terminal product of all three complement cascades, is a potent anaphylatoxin with immunomodulatory functions. A conformational peptide agonist of C5a, EP67, when chemically conjugated to a live attenuated fungal vaccine, selectively engaged C5aR on APCs, thereby promoting protective Th1 and Th17 responses while circumventing excessive inflammation otherwise mediated by neutrophil C5aR.¹¹⁶ Although these strategies employed chemi-

Table 5. Complement fragments as genetically engineered intramolecular adjuvants.

Intramolecular Adjuvant	Target Pathogen/Disease	Antigen	Fusion Construct Name(s)	Vaccine Delivery Platform	Adjuvant Mechanism	Experimental Model/Species	Ref.
C3d	SARS-CoV-2 (COVID-19)	Spike or RBD	Spike-C3d, RBD-C3d	mRNA-LNP vaccine	Lowered B-cell activation threshold, enhanced signaling	C57BL/6J mice; Ai14 reporter mice	111
	HIV-1	Env trimer (JRFL, 426c)	JRFL-L60-C3d	Recombinant Subunit	Enhanced germinal center formation & antibody response	C57BL/6 mice; VRC01gHL knock-in mice	112
C3b	SARS-CoV-2 (Omicron)	RBD	RBD-mC3	mRNA-LNP vaccine	Enhanced antigen presentation via CR1 on B cells/FDCs	C57BL/6 mice	114

Abbreviations: CR1, complement receptor 1; FDC, follicular dendritic cell; mC3, murine C3b; RBD, receptor-binding domain (of the SARS-CoV-2 spike protein).

cal conjugation rather than direct genetic fusion, they highlight the adjuvant value of the C1q-CD91 and C5a-C5aR signaling axes, offering important insights for future development of novel intramolecular adjuvant modules through genetic engineering approaches.

C3d-based complement fragments bridge B cell receptors and CD21 to facilitate B cell activation, antibody affinity maturation and memory B cell development, while sustaining robust humoral immune responses. Such adjuvants are particularly suitable for pathogens where protective efficacy depends on persistent high-titer neutralizing antibodies. However, substantial genetic and functional differences exist between species in complement components, such as the C3d–CR2 interaction between humans and mice,¹¹⁷ posing a major challenge to the clinical translation of these adjuvants.

Other intramolecular adjuvants

Immunoglobulin G Fc fragment. Fusion with the Fc fragment represents an effective strategy to address the short half-life of cytokines, aiming not only to enhance adjuvant activity but also to achieve precise pharmacokinetic modulation. Fc fusion proteins are chimeric molecules created by genetically linking an antigen to the Fc region of immunoglobulin G, resulting in self-advanting entities. The core mechanism of Fc as an intramolecular adjuvant lies in its natural interactions with the immune system, enabling targeted antigen delivery and synergistic immune activation. First, the Fc fragment binds to Fcγ receptors (FcγRs) on APCs, triggering efficient receptor-mediated endocytosis that directly delivers the antigen into intracellular processing compartments,¹¹⁸ enhancing antigen presentation efficiency by orders of magnitude.¹¹⁹ Simultaneously, binding to the neonatal Fc receptor (FcRn)¹²⁰ protects the fusion protein from lysosomal degradation and recycles it back into the circulation, significantly extending its *in vivo* half-life and creating a sustained release effect that prolongs immune stimulation. Notably, the Fc fragment also promotes cross-presentation via MHC I, effectively activating CD8⁺ T cell responses.¹²¹ This multi-level synergy makes Fc fusion an ideal intramolecular adjuvant platform, achieving coordinated antigen delivery, immune activation, and durable protection. In practice, the Fc fusion strategy has been successfully applied in vaccines against several major pathogens. An Fc-fused RBD domain of SARS-CoV-2 elicited high-level neutralizing antibodies and a Th1-biased response in mice and non-human primates.^{122,123} In HIV vaccine research, a gp120-Fc fusion remarkably enhanced the breadth and potency of antibody responses.¹²⁴ This approach has also shown considerable potential in tuberculosis^{119,125} and influenza vaccines.¹²⁶

Engineering efforts have further optimized Fc fusion proteins for improved performance. Point mutations such as YTE,^{127,128} REW,¹⁰ and others¹²⁹ markedly extend half-life, while alterations like S239D/I332E¹³⁰ enhance binding to activating FcγRs. From a production standpoint, the specific binding between Fc and Protein A/G simplifies purification without requiring additional tags. These optimizations improve immunogenicity and facilitate scalable manufacturing. With the advent of AI-assisted design tools, future efforts may enable precise programming of Fc fusion functions.

Heat shock protein GP96. GP96/GRP94, a key member of the heat shock protein 90 (HSP90) family, serves as a core molecular chaperone in the endoplasmic reticulum and plays an important role in antigen presentation and

immune activation. In a recent study, an AI-assisted protein design strategy was used to fuse the SARS-CoV-2 RBD antigen and the HPV L1 protein separately to the N-terminus of GP96, yielding stable antigen-GP96 fusion proteins with enhanced immunogenicity. For molecular design, AlphaFold3 was employed to screen and simulate a large number of binding modes between GP96 and different antigens. Based on this, dimeric fusion proteins with dual-terminal assembly were constructed. This configuration significantly increases the structural rigidity of the complex, promoting the formation of compact, nanoscale particles that effectively protect antigenic epitopes and enhance protease resistance. The design fully exploits the natural adjuvant function of GP96, which specifically binds to the low-density lipoprotein receptor-related protein 1 (LRP1) receptor on follicular dendritic cells, markedly prolonging antigen retention in lymph nodes. Consequently, it induces superior neutralizing antibody titers, durable memory B-cell responses, and broad CD8⁺ T-cell immunity compared to conventional adjuvants.¹³¹

Metallothionein-3 (MT3). MT3 is a member of the metallothionein family, characterized by its high cysteine content and metal-binding capabilities, particularly with zinc and copper. When fused to *Brucella* outer membrane protein Omp19 or the tetanus toxin Hc fragment, it significantly enhances immunogenicity. Notably, compared to conventional adjuvants, the MT3 fusion protein induces high-titer specific antibodies within 7 days post-immunization and elicits a potent, rapid, and durable immune response even at low doses.¹³² The precise immunological mechanisms underlying the adjuvant activity of MT3, however, remain incompletely understood, potentially linked to its sulfur cluster configuration. Further studies are needed to elucidate its roles in immune cell activation and signaling pathways.

Beyond the types previously discussed, various other intramolecular adjuvants also show potential, such as the natural tetrapeptide Tuftsin,¹³³ which activates macrophage and neutrophil functions, the PD1 splice variant Δ42PD1, which enhances T-cell immunity,¹³⁴ and the bee-venom phospholipase A2 mutant bvPLA2m, which operates through a membrane-anchored delivery system.¹³⁵ Together, these tools substantially expand the molecular toolkit, enabling customized immune programs for pathogens refractory to conventional adjuvant approaches (Table 6).

Finally, it is worth noting that genetic fusion and chemical conjugation are not mutually exclusive. Emerging hybrid strategies that combine recombinant protein scaffolds with site-specific conjugation of non-proteinaceous ligands can circumvent the biosynthetic limitations of genetic encoding while retaining the molecular homogeneity and scalable production advantages of recombinant systems, thereby further broadening the design space of intramolecular adjuvants. As bioconjugation studies summarize, diverse semisynthetic platforms enable site-selective covalent linkage of imidazoquinoline TLR7/8 agonists to folded recombinant antigens via controllable ligation; such conjugates greatly amplify antigen-specific humoral immunity by co-delivering antigen and adjuvant to identical antigen-presenting cells.¹³⁶ Similarly, genetically encoded incorporation of unnatural amino acids bearing bioorthogonal handles into recombinant proteins has enabled the precise and homogeneous attachment of STING agonists and other immunomodulatory small molecules, as shown in recent protein engineering studies.¹³⁷ These examples illustrate how the convergence of recombinant precision and

Table 6. Other molecules as genetically engineered intramolecular adjuvants.

Intramolecular Adjuvant	Target Pathogen/Disease	Antigen	Fusion Construct Name(s)	Vaccine Delivery Platform	Adjuvant Mechanism	Experimental Model/Species	Ref.	
Fc domain	human IgG1	SARS-CoV-2 (COVID-19)	RBD (aa 331-524)	RBD-Fc	Recombinant Subunit	Enhanced antigen presentation, extended half-life	Cynomolgus macaques; BALB/c mice; hACE2 transgenic mice	122,123
	Mouse IgG2a Fc	HIV-1	gp120	gp120-Fc	DNA vaccine	Enhanced antigen presentation, extended half-life	BALB/c mice	124
	Mouse IgG2a Fc	<i>Mycobacterium tuberculosis</i>	CFP-10	CFP-10:Fcy2	Recombinant Subunit	Enhanced antigen presentation, extended half-life	BALB/c mice	125
	human IgG1	Influenza A Virus	Hemagglutinin (HA)	HA-HuFc	Recombinant Subunit	Enhanced antigen presentation, extended half-life	BALB/c mice	126
Heat shock protein GP96/GRP94	SARS-CoV-2 (COVID-19); HPV	RBD (aa 319-545); L1 (81-535aa), E7	RBD-GP96-Fusion; L1-GP96-Fusion, E7-GP96-Fusion	Recombinant Subunit	Targeted antigen retention (via LRP1 on FDCs), enhanced germinal center activation	BALB/c mice; BALB/c nude mice; HLA-A2 transgenic mice; hACE2 transgenic mice	131	
Metallothionein-3 (MT3)	<i>Brucella abortus</i> , <i>C. tetani</i>	Omp19, Hc	MT3-Omp19, MT3-Hc	Recombinant Subunit	Rapid antibody response, early germinal center reaction	BALB/c mice	132	
Tuftsia	Transmissible Gastroenteritis Virus	Spike protein D site	20MT6D, 40MT6D	Recombinant Subunit	Activated mucosal and systemic immune responses	BALB/c mice	133	
Δ42PD1	HIV-1	Gag p24	msΔ42PD1-p24Fc	DNA vaccine	Synergistically enhanced Th1/Th2 antibody responses	BALB/c mice; Human PBMCs	134	
bvPLA2m	NY-ESO-1 ⁺ Tumors	NY-ESO-1 antigen	bvPLA2m-NY-ESO-1 (PNY)	Recombinant Subunit	Enhanced membrane binding and cross-presentation	Human PBMCs; Human monocyte-derived DCs	135	

Abbreviations: $\Delta 42PD1$, alternatively spliced isoform of programmed cell death protein 1; CFP-10, culture filtrate protein 10 (from *Mycobacterium tuberculosis*); Fc, fragment crystallizable (region of antibody); Hc, heavy chain (domain of tetanus neurotoxin); RBD, receptor-binding domain (of the SARS-CoV-2 spike protein); LRP1, low-density lipoprotein receptor-related protein 1; FDC, Follicular dendritic cell. Bacterial genus and species names are italicized in the 'Target Pathogen/Disease' column.

chemical versatility can overcome the intrinsic constraints of purely genetic or purely chemical approaches.

RESEARCH AND CLINICAL CHALLENGES OF INTRAMOLECULAR ADJUVANTS

Although intramolecular adjuvants have shown considerable potential in preclinical studies, their translational development faces a distinct set of challenges that span structural design, immunological compatibility, and clinical translation and manufacturing.

Structural design challenges: maintaining conformational integrity and product homogeneity

Preserving the native conformation of antigen and adjuvant domains is essential for functional intramolecular adjuvant vaccines, as genetic fusion may trigger protein misfolding, interdomain interference, epitope masking and junction cleavage that impair immunogenicity.¹³⁸ Domain fusion can disrupt individual folding processes and produce nonfunctional misfolded aggregates. For instance, CTB requires stable pentameric self-assembly for adjuvant activity, while inappropriate antigen fusion may cause steric hindrance and block assembly.²¹ Linker design thus serves as a core opti-

mization strategy to offer structural flexibility, enable independent domain folding and alleviate steric conflicts.¹³⁹ Flexible glycine-rich linkers such as $(G_4S)_n$ are most widely used, yet they raise risks of proteolysis and domain swapping-induced aggregation. By contrast, rigid $(EAAAK)_n$ linkers fix spatial orientation and reduce assembly steric hindrance, benefiting adjuvants dependent on conserved quaternary structures. However, rigid linkers may mask key epitopes and block immune recognition, limiting their application in intramolecular adjuvant design. Cleavable linkers containing protease sites can be specifically processed *in vivo*, achieving spatiotemporal separation of antigens and adjuvants and directing them to distinct subcellular immune compartments.¹³⁹ Though underutilized currently, cleavable linkers possess great potential for rational vaccine engineering (Table S1). Beyond linker design, structural heterogeneity including variable glycosylation, truncated proteins, disulfide mismatches and high-molecular-weight aggregates also compromises the stability and immunogenicity of fusion proteins, requiring strict process control.

Immunological challenges: balancing efficacy and safety

At the immunological level, the design of intramolecular adjuvants confronts a trilemma: maximizing immunogenicity, minimizing abnormal

immune activation, and accommodating population diversity. The physical linkage of foreign antigens to self-proteins or potent immune stimulants creates unique safety liabilities that must be rigorously addressed.

Minimizing abnormal immune activation. Many intramolecular adjuvants rely on robust PRR activation to boost immunity, yet overactive TLR4/TLR5 signaling may trigger severe systemic toxicity. High-dose flagellin or potent TLR4 agonists drive excessive NF- κ B/MAPK cascade activation, inducing cytokine release syndrome marked by acute tissue injury, neutrophil infiltration and autophagic dysfunction.⁷⁹ As typical DAMPs with powerful chaperone activity, HSPs like GP96 can overstimulate innate immunity and potentially aggravate autoimmune lesions via molecular mimicry without precise regulation. Another prominent safety hazard stems from disrupted self-tolerance when endogenous cytokines are fused with antigens: GM-CSF-antigen fusions may expose hidden neoepitopes to elicit high levels of anti-GM-CSF neutralizing antibodies and blunt CD8⁺ T cell responses,⁹⁸ while fused interferons or interleukins might redirect T helper signals toward self-cytokines and trigger epitope spreading that disturbs immune homeostasis. Co-expression systems with separate antigen and cytokine cistrons effectively alleviate this risk by retaining intact cytokine conformation and shielding cryptic self-epitopes at fusion junctions. Besides, pre-existing carrier-derived T cell help comes with the risk of CIES: conventional diphtheria and tetanus toxoids with strong inherent immunogenicity readily produce high-titer carrier antibodies to suppress neoantigen responses, whereas modified carriers such as CRM197 deliver far weaker inhibitory effects.⁵⁵ Such suppression is dose-dependent and tightly controlled by carrier structural design; moderate antigen modification and proper conjugation density balance carrier-antigen immunodominance and help avoid biased immune responses.^{140,141} Collectively, rational low-CIES carrier selection, strict dosage control, optimized immunization schedules and AI-assisted structural design help confine carrier-mediated immune suppression to a manageable range, fully exploiting the benefits of pre-existing immune memory while generating potent protective immunity against target neoantigens.

Inter-individual and inter-species variability. A fundamental concern is species-specific differences in innate immune signaling, which often cause promising preclinical observations in mice to fail when evaluated in humans. For instance, the C3d-CR2 interaction, a cornerstone of complement-based adjuvant design, exhibits marked divergence between mice and humans, raising concerns about the translatability of vaccine efficacy data from small-animal models.¹¹⁷ Likewise, TLR expression profiles and ligand specificities differ across species, requiring careful evaluation of the predictive value of animal models during preclinical development. For T cell epitope-based adjuvants, the extensive polymorphism of human MHC (HLA) molecules poses another major hurdle. Carriers such as tetanus toxoid-derived P2 and P30 provide universal T-helper epitopes; however, distinct HLA haplotypes present variable peptide repertoires, leading to inter-individual variability in response magnitude and restricting the breadth of population coverage.

The strategic challenge of immune polarization, matching Th1- or Th2-polarizing adjuvants to specific pathogen classes, further intersects with these manufacturing and regulatory considerations. For intracellular pathogens, Th1-polarizing adjuvants, such as type I interferon (IFN α), are required to activate cellular immunity. For extracellular bacteria or toxins, Th2-polarizing adjuvants such as CTB can effectively enhance humoral immunity. This matching must also account for population differences, such as immune senescence in the elderly or immaturity in infants.

Safety of toxin derivatives. Although bacterial toxin derivatives are genetically detoxified, residual safety concerns persist. Their high-affinity binding to ubiquitous receptors may interfere with normal cellular signaling pathways or lead to unintended cross-reactivity. Moreover, the presence of pre-existing anti-toxin antibodies can not only cause CIES but also trigger hypersensitivity reactions upon re-exposure. Therefore, the rational design of intramolecular adjuvants must integrate de-immunization algorithms to remove T-cell epitopes from self-proteins and employ cleavable linkers to separate functional domains *in vivo*, thereby decoupling potent adjuvant activity from autoimmune and toxic liabilities.

Translational and regulatory challenges: CMC requirements and classification

Intramolecular adjuvant fusion vaccines are subject to strict FDA and EMA Chemistry, Manufacturing, and Controls (CMC) requirements throughout clinical approval. Regarded as new molecular entities, these fusion products may require comprehensive toxicological, pharmacokinetic and immunogenicity evaluations. The 2026 FDA CP 7346.832M clarifies inspection standards for CDER-regulated Fc-fused and cytokine-fusion therapeutic proteins, progressively improving standardized regulation for intramolecular adjuvant products. Driven by optimized regulatory frameworks, multiple candidates have entered advanced clinical stages or obtained marketing authorization (Table 7). Approved vaccines Sipuleucel-T¹⁴² and V-01⁸⁷ have proven the clinical feasibility of intramolecular adjuvant design, while influenza, tumor and malaria vaccine candidates have completed Phase I/II trials with satisfactory safety and immunogenicity. During production, fusion protein expression quality can be influenced by culture systems and protein intrinsic characteristics, potentially causing aggregation and inclusion body formation, which necessitates targeted process optimization. Uniform glycosylation patterns and batch consistency require strict supervision, and viral vector vaccines need standardized detection of residual DNA and enzyme activity. While these manufacturing challenges are common for recombinant vaccines, systematic quality assessment may help ensure the safety and stability of intramolecular adjuvant vaccine products.

AI-DRIVEN SOLUTIONS FOR INTRAMOLECULAR ADJUVANT DEVELOPMENT

AI offers a powerful suite of computational tools to systematically overcome these challenges, advancing vaccine development from an experience-driven process toward a predictive and programmable immunological paradigm.

Sequence-level screening: mining bioactive peptides for adjuvant candidates

AI-based sequence screening alleviates the key limitation of efficient discovery of novel genetically encodable adjuvants. While numerous tools support epitope prediction,^{143,144} professional platforms for adjuvanticity screening remain scarce, as adjuvant functional sequences differ greatly from conventional antigen epitopes. Intramolecular adjuvant design also requires candidate sequences to adapt to fusion protein structures, bringing additional design challenges. Pioneering studies have established an AI-driven "Mine-Simulate-Screen" strategy for adjuvant development. The VaxinPAD platform achieves 95% accuracy in predicting APC-activating peptides through machine learning training.¹⁴⁵ It has been applied to screen arthropod antimicrobial peptides, obtaining multiple low-toxicity adjuvant candidates via multi-index filtering and TLR docking verification.¹⁴⁶ Advanced deep learning models can simulate and predict the complex immunological outcomes elicited by a given adjuvant. A recent transcriptome-based deep learning model can predict adjuvant-triggered antibody responses with 96.9% accuracy, and identify core immune pathways determining different adjuvant functions.¹⁴⁷ The integration of these two approaches, mining for novel candidates and simulating their immunological impact, points toward a refined screening paradigm for molecular adjuvant design.

Structure-level design: predicting complexes and guiding linker optimization

AI structural tools such as AlphaFold3 assist in resolving key structural obstacles in intramolecular adjuvant design, helping maintain the stable folding and independent function of fused antigen and adjuvant domains.¹⁴⁸⁻¹⁵⁰ *In silico* structural simulation enables pre-experimental assessment of protein steric clashes and epitope masking, with PAE analysis acting as a critical criterion for judging domain flexibility and rigidity. Discrepant PAE values between intra-domain and inter-domain regions may indicate flexible linker-connected independent folding, whereas consistent low PAE across the whole construct tends to form rigid domain arrangement. Different structural characteristics can guide targeted linker selection: rigid structures are suitable for multimeric adjuvants including CTB,²¹ while flexible linkers may benefit antigens and adjuvants requiring separate receptor binding. AlphaFold3-assisted structural optimization has been practically verified in GP96 fusion vaccine design, which improves conformational stability and epitope expo-

Table 7. Representative intramolecular adjuvant vaccines (genetically fused) that have entered clinical trials.

Vaccine Name	Antigen–Adjuvant Genetic Fusion Design	Target Indication	Developer	Clinical Phase	Key Findings	Trial ID
I . Fc-Fusion						
V-01	IFN α -PADRE-RBD-Fc dimer fusion (CHO)	COVID-19	Livzon Pharmaceutical, China	Emergency use authorization (China, 2022); Phase 3	Durable neutralizing Ab & Th1-biased T-cell responses; non-inferior to mRNA boosters in Phase 3	NCT05096832; NCT05096845
AKS-452	RBD–Fc fusion (CHO, thermostable)	COVID-19	Akston Biosciences/UMCG	Phase 1/2	Robust neutralizing Ab after single dose	NCT04681092; NCT05124483
Baiya VAX1/ VAX 2	Plant-produced RBD–Fc fusion	COVID-19	Baiya Phytopharm/Chula Univ.	Phase 1	First plant-derived Fc-fusion COVID vaccine in clinic; low-cost production (VAX 2 unpublished)	NCT04953078; NCT05197712
II . Cytokine Genetic Fusion						
Sipuleucel-T (PA2024)	PAP–GM-CSF single-chain fusion (baculovirus); used <i>ex vivo</i> to pulse autologous PBMCs	mCRPC (prostate cancer)	Dendreon Corp., USA	FDA approved (2010); Phase 3	4.1-mo median OS improvement (HR = 0.78); first FDA-approved therapeutic cancer vaccine	NCT00065442
V-01	IFN α -PADRE-RBD-Fc dimer fusion (CHO)	COVID-19	Livzon Pharmaceutical, China	Emergency use authorization (China, 2022); Phase 3	Durable neutralizing Ab & Th1-biased T-cell responses; non-inferior to mRNA boosters in Phase 3	NCT05096832; NCT05096845
III. Flagellin (TLR5) Genetic Fusion						
VAX102 (STF2.4×M2e)	Flagellin (STF2)–four tandem M2e peptides	Influenza (seasonal/pandemic)	VaxInnate, USA	Phase 1	Well-tolerated at $\leq 1 \mu\text{g}$; M2e-specific IgG induced	NCT00603811 (PMID: 21624416)
VAX125 (STF2.HA1-SI)	Flagellin (STF2)–HA1 domain (H1N1)	Influenza (elderly ≥ 65 years)	VaxInnate, USA	Phase 2	>10 -fold HAI titer increase at $5 \mu\text{g}$ in elderly; overcame immunosenescence	NCT00966238
VAX161 (STF2.HA5 H5N1)	Flagellin (STF2)–HA1 domain (H5N1)	Avian influenza (H5N1)	VaxInnate, USA	Phase 1	No Results Posted	NCT01560793
IV . Tetanus Toxin Fragment C/T–Helper Epitope Genetic Fusion						
p.DOM-PSMA27	DNA vaccine: Fragment C (DOM)–PSMA peptide fusion	Prostate cancer	Univ. of Southampton	Phase 1/2	PSMA-specific CD8 $^{+}$ T-cell responses detected in 16/30 patients; increased PSA doubling time	(PMID: 22729556)
p.DOM-WT1-37	DNA vaccine: DOM–WT1 peptide fusion	WT1 $^{+}$ hematological malignancies	Univ. of Southampton	Phase 2	Induced WT1-specific CD8 $^{+}$ T-cell responses in patients with WT1-positive hematological malignancies	NCT01334060
ICC-1132 (Malarivax)	HBcAg VLP: CSP B-cell + P2/P30 T-helper epitopes inserted into HBc loop	Malaria (<i>P. falciparum</i>)	Apovia	Phase 1	Safe; induced anti-CSP Ab and T-cell responses	(PMID: 15501783/15908388)

Abbreviations: Ab, antibody; CHO, Chinese hamster ovary; CSP, circumsporozoite protein; HA, hemagglutinin; HAI, hemagglutination inhibition; HBcAg, hepatitis B core antigen; HR, hazard ratio; mCRPC, metastatic castration-resistant prostate cancer; M2e, matrix protein 2 ectodomain; OS, overall survival; PBMC, peripheral blood mononuclear cell; PSA, prostate-specific antigen; VLP, virus-like particle; WT1, Wilms tumor 1.

sure through rational antigen docking.¹³¹ This strategy may convert traditional trial-and-error linker modification into efficient computer-aided structural optimization, greatly advancing the rational design of intramolecular adjuvant vaccines.

De novo design: creating customized adjuvants with generative AI

Generative AI enables the *de novo* creation of functional-customized adjuvants, going beyond traditional natural sequence optimization and facilitating

the development of programmable immune modulation.^{151,152} Computational design approaches support the engineering of artificial immune modulators. Artificially designed TLR3 miniproteins generated via Rosetta RigidBody can bind human TLR3 with high affinity and activate NF- κ B signaling in multivalent forms, confirming the feasibility of *de novo* PRR agonist design.¹⁵³ Modular domain assembly allows precise immune signal regulation and independent functional optimization, which is well-suited for customized intramolecular adjuvant engineering.^{154,155} The integration of minibinder design and

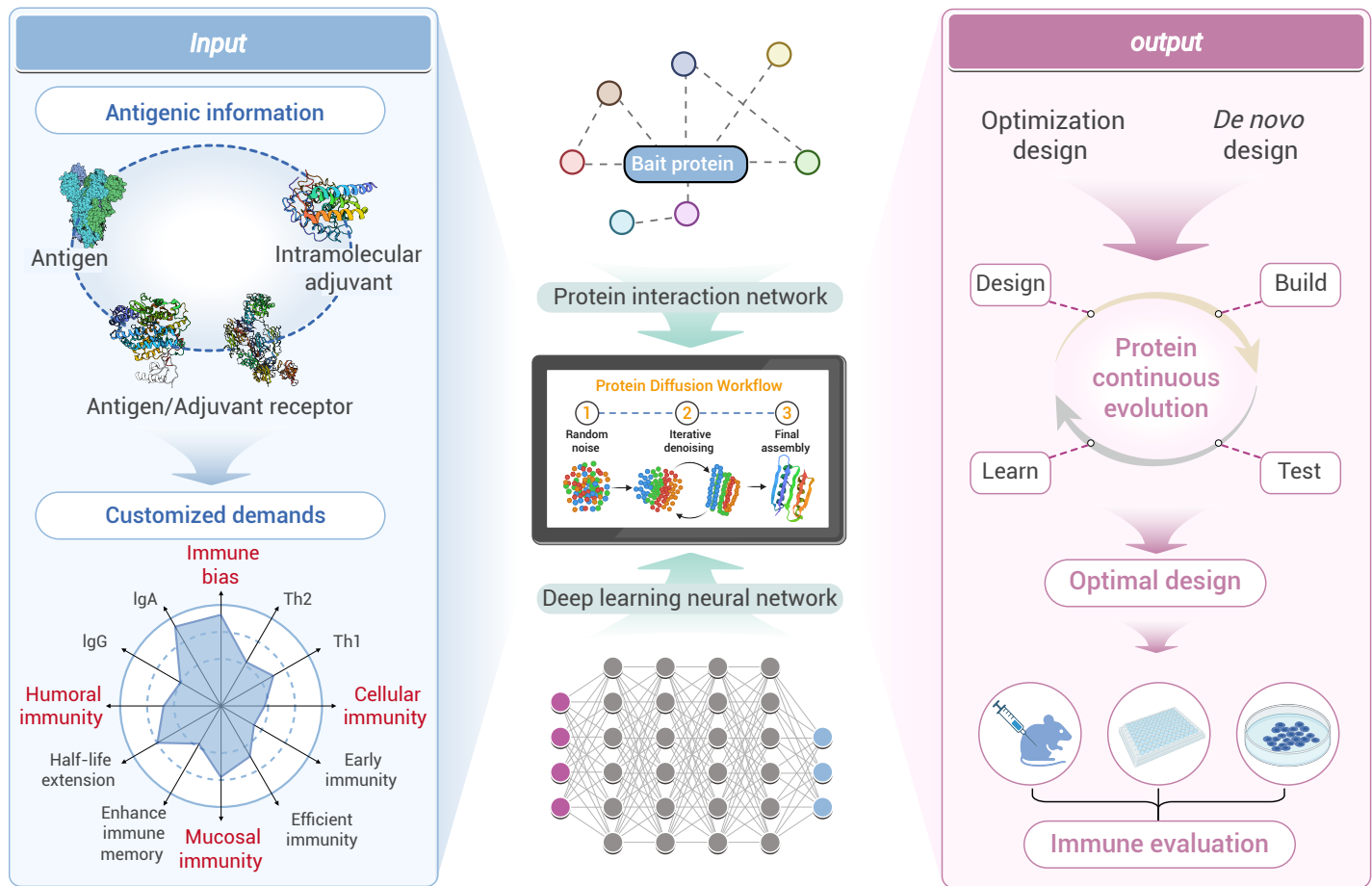


Figure 3. AI-driven intelligent design platform for intramolecular adjuvants. This generative artificial intelligence platform, centered on novel algorithms such as diffusion generative models, leverages a dedicated intramolecular adjuvant database to analyze antigen–adjuvant–receptor interactions and specific immune response requirements. It establishes a closed-loop pipeline spanning computational design, structural optimization, and functional validation.

modular assembly lays a solid foundation for constructing diversified adjuvant libraries with customizable activation characteristics. Modern generative pipelines integrate RFdiffusion-based scaffold generation,^{151,156} Protein-MPNN-assisted sequence optimization and high-throughput screening to obtain high-quality adjuvant candidates.¹⁵⁷ Iterative design-build-test-learn cycles help align computational prediction with biological function, optimizing adjuvant potency, stability and safety through continuous model iteration (Figure 3).^{157,158}

CONCLUSION

Genetic engineering-driven intramolecular adjuvant strategies have transcended the limitations of conventional vaccine design, representing a transformative approach to enhancing vaccine immunogenicity through multi-level synergistic immunomodulation encompassing targeted delivery, signal activation, and immune polarization. Compared with traditional physical mixture approaches, genetically fused intramolecular adjuvants offer four distinct advantages (Table S2): precise intramolecular collocation to reduce epitope masking, modular multi-epitope assembly for controllable antigen-adjuvant stoichiometry, optimized protein stability via codon engineering, and synchronized APC co-delivery to maximize immune synergy. This fusion platform delivers superior molecular homogeneity and design precision, with inherent compatibility to nucleic acid and viral vector vaccines, underpinning the vision of programmable immunity. However, this ideal paradigm is still restricted by interspecies signaling divergence, human HLA polymorphism and carrier-induced epitope suppression.

Although numerous fusion vaccine candidates have yielded promising preclinical outcomes and some have entered clinical trials or obtained marketing approval (Table 7), their translation is hindered by structural, immunological and regulatory barriers. Developers need to balance native

domain conformation with batch consistency, control aberrant innate immune activation and CIES risks, and fulfill rigorous CMC, toxicological and immunological evaluation requirements. Current ameliorative strategies such as multi-adjuvant coordination,¹⁵⁹ ferritin-based self-assembling protein nanoparticles (SAPNs)¹⁶⁰ and mRNA-LNP lymph-targeted delivery¹⁶¹ have been proposed, yet multiple core bottlenecks across the whole research pipeline await resolution.

AI reshapes intramolecular adjuvant research by shifting empirical trial-and-error to predictive rational design, consisting of three complementary technical modules: sequence mining via integrated Mine-Simulate-Screen pipelines, AlphaFold3-assisted structural optimization guided by PAE analysis,¹⁶² and generative *de novo* adjuvant construction based on RFdiffusion and Protein-MPNN. These technologies form closed design-build-test-learn loops that support full-cycle simulation from epitope screening to immune response prediction. *De novo* adjuvant engineering is still in the preliminary phase, yet iterative upgrades of generative algorithms and high-throughput experimental validation will profoundly accelerate vaccine development, lower research costs and improve the clinical success rate of next-generation intramolecular adjuvant vaccines, laying a solid foundation for rapid development of vaccines against emerging and complex pathogens.

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AUTHOR CONTRIBUTIONS

Y.W. and W.L. conceptualized and designed the review and drafted the manuscript. Z.Z. and T.W. contributed to figure preparation and participated in manuscript discussions. J.X., X.Z. and J.Z. supervised the work and revised the manuscript. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

No new data were created or analyzed in this study.

SUPPLEMENTAL INFORMATION

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