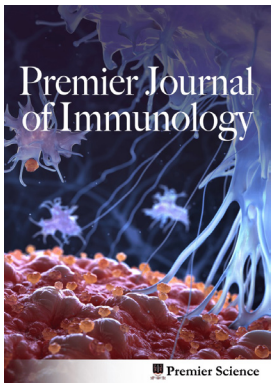

Combinatorial Toll-like Receptor Agonists: Advancing Immune Modulation for Global Health Challenges

Jainu Ajit

ABSTRACT

Toll-like receptors (TLRs) are essential pattern recognition receptors that initiate innate immune responses and shape adaptive immunity. While individual TLR agonists have been extensively used as vaccine adjuvants and immunomodulators, recent advancements highlight the potential of concurrently activating multiple TLRs to mimic natural infections and generate more effective synergistic immune responses. This review examines the molecular and cellular principles underlying multi-TLR activation, including receptor localization, signaling crosstalk, ligand affinity, and temporal dynamics. Additionally, the review explores recent strategies for delivering combinatorial TLR agonists along with their clinical implications. A deeper mechanistic understanding of combinatorial TLR engagement could pave the way for the rational design of next-generation adjuvants and personalized immunotherapies for infectious diseases, cancer, and beyond.

Keywords: Toll-like receptors, Combinatorial TLR agonists, Immune modulation, Vaccine adjuvants, Synergistic immune responses

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Introduction

An effective immune response occurs when immune cells detect and respond to pathogens and other danger signals in their environment. One class of receptors that enables this protection is toll-like receptors (TLRs). TLRs represent the most prominent family of pattern recognition receptors, and their activation is the first step in inducing an immune response.[1,2] Agonists targeting TLRs elicit unique immune reactions that can be tailored to enhance immediate protection against various diseases, including cancers, and to develop improved vaccines against subsequent threats. Moreover, the simultaneous activation of multiple TLRs yields unique immune reactions by mimicking natural infections from viruses, bacteria, fungi, and other pathogens, resulting in an improved response compared to single TLR activation (see Figure 1 for a detailed list of TLR agonists in various pathogens). Upon activation, innate immune cells such as dendritic cells (DCs) and macrophages upregulate costimulatory molecules and secrete pro-inflammatory cytokines to prime adaptive immunity. An effective T-cell and B-cell response is key to host defense responses and protection against pathogens. Therefore, modulating TLR activation and creating novel adjuvants is fundamental to advancing global health.

To understand how combinatorial TLR agonists can be rationally designed for use as effective adjuvants, one must examine a few key features, which include the molecular and cellular characteristics that govern synergistic TLR activation. This fundamental understanding provides the foundation for defining design parameters that influence how combinatorial agonists can be formulated and delivered to immune cells. They include parameters such as the structure, location, and signaling mechanisms of TLRs, as well as the affinity and stoichiometry of multiple TLR agonists, and the spatio-temporal features of activation. Often, several of these factors influence the trajectory of downstream immune activation resulting from multi-TLR activation. This review also highlights co-delivery approaches explored thus far and their implications in addressing health challenges, from infectious diseases to cancer immunotherapy. The use of Artificial Intelligence (AI) and computational design for novel TLR agonists, along with challenges related to varying TLR expression, are also addressed to encompass future perspectives and challenges.

Molecular and Cellular Characteristics Governing TLR Activation

Structure and Location of TLRs

TLRs are type-1 transmembrane proteins with a similar structural organization. The N-terminal ectodomain contains leucine-rich repeats, followed by a transmembrane domain, and then the cytosolic terminal has a Toll/interleukin 1 (IL-1) receptor (TIR) domain.[3] The N-terminal domains adopt a horseshoe-shaped structure, which, after binding to their agonist, forms a homo or heterodimer. Dimerization leads to downstream signaling via various adaptor proteins, resulting in immune activation.[4]

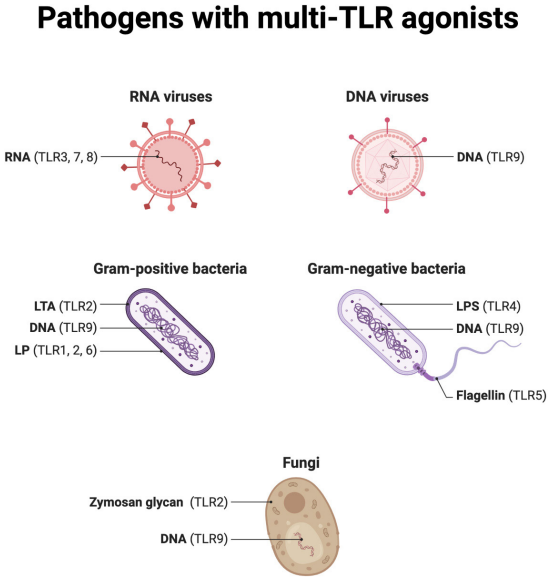

Fig 1 | Pathogens exhibit a combination of ligands that simultaneously activate multiple TLRs (created using BioRender)

While TLRs are synthesized in the endoplasmic reticulum (ER), they are directed to the plasma or endosomal membranes to sense various pathogen-associated molecular patterns (PAMPs). A protein called UNC93B1 controls the trafficking of intracellular TLRs to the endosomal membrane.[5] Another protein, PRAT4, is also involved in the trafficking of other TLRs, including TLR1, TLR2, TLR4, TLR7, and TLR9, from the ER to their respective locations.[6] Plasma membrane-localized TLRs detect cell surface components of microbes, whereas endosomal TLRs detect their nucleic acids. In humans, TLRs respond to diverse PAMPs, including bacterial flagellin (TLR5), viral dsRNA (TLR3), CpG-unmethylated DNA (TLR9), etc.

TLR Signaling

The cytoplasmic TIR domain is responsible for initiating downstream signaling pathways. Each TLR recruits TIR-domain-containing adaptors such as Myeloid Differentiation Primary Response Gene 88 (MyD88), TIR domain-containing adaptor protein (TRIF), TIR domain-containing adaptor protein (TIRAP), or TRIF-related adaptor molecule (TRAM).[7] Therefore, the simultaneous activation of different TLRs yields distinct signaling cascades, and a brief understanding of these mechanisms enables us to appreciate better and understand combinatorial TLR activation.

Except for TLR3, all other TLRs utilize MyD88 to activate nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPKs), releasing inflammatory cytokines. In unstimulated cells, inactive NF-κB is found in the cytoplasm due to its interaction with proteins known as inhibitor of NF-κB (IκB). However, stimulation with TLR agonists leads to the translocation of NF-κB to the nucleus.[8] TRIF is utilized by TLR3 and TLR4, promoting an alternative pathway activating IRF3, NF-κB, and MAPKs to induce type I interferon (IFN) and inflammatory cytokine genes.[9] TIRAP is an adaptor protein that is downstream of TLR4 and TLR2.[10] In TLR4 signaling, TIRAP interacts with TRAF6 to initiate NF-κB activation.[11] An adaptor molecule called TRAM is crucial in the TLR4-mediated MyD88-independent signaling pathway.[12] Since multiple reviews provide vivid descriptions of TLR signaling pathways, this manuscript will not discuss them in detail.[3,4,7,9,13] A detailed list of some TLRs, their cellular location, and downstream immune effects is provided in Table 1.

Generally, the simultaneous stimulation of TLRs that signal through different pathways has been shown to induce synergy.[14] However, studies also indicate that multi-TLR agonists targeting the same pathway, following MYD88 activation, upregulate cytokine production in bone-marrow-derived DCs (BMDCs).[15] Apart from the combinations used, the timing of activation also influences downstream signaling, for example, the sequential administration of MyD88-dependent and MyD88-independent pathways induces priming.[16] Significant work by Pandey and colleagues has demonstrated a combinatorial logic governing the stimulation of multi-TLR agonists in mouse and human cells.[17] The authors observed that single and dual TLR agonists dictate the effects of using three agonists simultaneously, and this occurs through the regulation of transcription, chromatin restructuring, and protein synthesis.

NF-kb Switch Response

Following a natural infection, immune cells are often activated simultaneously by multiple PAMPs. Regardless of the specific signaling adaptors that different TLRs utilize, they all converge on NF-κB activation and downstream inflammation. TNF-α is one such inflammatory cytokine that is increasingly secreted following NF-κB activation; however, cells experience varying levels of this cytokine due to random fluctuations in the cellular environment, regardless of infection. This necessitates a mechanism by which cells can sense the difference in inflammatory cytokine levels, such as TNF-α, in the event of an actual immune response versus noise.

Table 1 | List of the most common TLRs, their cellular expression, ligands, and immune activation pathways and effects

TLR	Expression	Ligands	Signaling Pathway	Immune Effects
2	Plasma Membrane	Lipopeptides, Peptidoglycan, Lipoteichoic acid, Zymosan, Pam2Cys, Pam3Cys	MyD88	Inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12) CD80, CD86 upregulation
3	Endolysosome	dsRNA, polyIC	TRIF	IFNβ CXCL10 CD80, CD86 upregulation
4	Plasma Membrane	LPS, Monophosphoryl lipid A (MPLA)	MyD88, TRIF	Inflammatory cytokines (TNFα,IL-1β,IL-6) IFNβ CXCL10 CD80, CD86 upregulation
5	Plasma Membrane	Flagellin	MyD88	Inflammatory cytokines (TNFα,IL-1β,IL-6,IL-8) CD80, CD86 upregulation
7	Endolysosome	ssRNA, Resiquimod	MyD88	Type 1 IFN CD80, CD86 upregulation
8	Endolysosome	ssRNA, Resiquimod	MyD88	inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12) CD80, CD86 upregulation
9	Endolysosome	CpG-DNA	MyD88	Type 1 IFN CD80, CD86 upregulation

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Kellogg et al. identified that NF-kb uses a switch-response to help cells filter out this background noise by integrating information about the intensity and the timing of the inflammatory signal in response to a singular TLR activation.[18] A detailed analysis by the same group revealed a non-integrative processing to explain how cells did not exhibit a combined response when simultaneously activated by TLR2 and TLR4. In this case, cells exhibited an NF-kb response that resembled either TLR2 or TLR4 activation.[19] Therefore, signaling pathways are crucial in forming downstream immune responses when combinatorial TLR agonists are used.

Affinity and Stoichiometry of TLR Dimer Binding to PAMPs

Apart from different adaptor molecules responsible for downstream signaling responses, TLRs, due to their unique structures and locations, also exhibit varying affinities and binding ratios to their respective ligands. For example, a single molecule of di-acylated lipoprotein binds to a TLR1/2 heterodimer, whereas two CpG molecules bind to a TLR9 homodimer.[1] Similarly, TLR4, 7, and 8 bind their cognate ligands with low affinity, whereas the rest of the TLRs interact with high affinity in the presence of low nanomolar concentrations of ligands. A detailed list of affinity and ratio of TLRs and their respective PAMPs are listed in Table 2. Therefore, combinatorial TLR activation may be modulated by controlling the number of activating ligands with appropriate binding affinities.

Spatio-Temporal Activation of PAMPs

In a natural infection, pathogens have a fixed structure that dictates the location of different TLR-activating agonists.[20] They are also capable of generating robust immune responses in the host. For example, in a herpes viral infection, TLR2 and TLR9 agonists exist at a particular molecular configuration. The yellow fever virus vaccine is highly successful due to its ability to activate multiple TLRs, such as TLRs 2, 7, 8, and 9, which elicit proinflammatory cytokines and interferon-alpha.[21] Similarly, a gram-negative bacterium simultaneously activates TLR4 via LPS, TLR5 via flagellin, and TLR9 via unmethylated CpG DNA. To generate successful vaccines, it is thus crucial to create a synergistic response by utilizing multiple TLR agonists in a single construct, such as polymers or small-molecule linkers. Work by Ryu and colleagues has explored this area, demonstrating that synergies indeed depend on the spatial organization of dual TLR agonists.[22] The authors demonstrated that varying the distance between two agonists by altering the linker length affects the level of NF-κB activity and the resulting inflammation. Similarly, steric hindrance prevents activation of TLRs in combinatorial systems with limited spatial flexibility.

A fixed spatial organization is further complemented by modulating the kinetics of TLR activation to induce a synergistic response. Combinatorial TLR agonist delivery ensures that all of the agonists are taken up by immune cells into the same endosome, unlike when delivered separately.[23] For example, Tom et al. demonstrated that a covalently linked tri-agonist targeting TLR 4, 7, and 9 increased activation of NF-κB and cytokine production compared to a mixture of unlinked agonists. Similarly, it is hypothesized that constructs containing multiple TLR agonists that bind to both surface and endosomal TLRs follow a sequential activation mechanism, leading to enhanced responses. Furthermore, TLR signaling pathways are being increasingly explored in detail, and many aspects remain unclear. For example, although TLR2 is a plasma membrane-associated TLR, research by Brandt and colleagues has highlighted that TLR2 agonists are internalized into endosomes for NF-kb activation in human monocytes.[24]

Summary

As mentioned previously, it is rare for a single factor to contribute to the immunogenicity of combinatorial TLR agonists. Often, multiple factors influence the trajectory of downstream immune responses. Kimani et al. detail how initial activation kinetics, ligand specificity, and agonist dosage influence the activity of dual-linked TLR systems *in vitro*.[25] As the complexity of the model organism increases, so do the contributing factors to immune activation. In this review, we present several examples of combinatorial TLR activation strategies and their clinical implications.

Combinatorial TLR Activation Strategies

Multiple TLR agonists can be delivered to innate immune cells using various strategies. These include a solution-based delivery approach, where unlinked mixtures are simultaneously co-delivered to cells or more complex animal models. By increasing organization and structural complexity, covalently linked constructs provide unique methods to target multiple TLRs simultaneously. In addition to this approach, nanoformulations are used to either surface conjugate or encapsulate various TLR agonists. Each of these strategies offers distinct features, from understanding the mechanistic details of synergy to targeting specific disease conditions. A hypothetical example of a dual TLR agonist targeting TLR4 and TLR7, using the three strategies, is depicted below in Figure 2.

Co-administration of Unlinked TLR Agonists

The easiest way to study the synergy between two TLR agonists is to deliver them in solution, either *in vitro* or *in vivo*. This method offers a unique advantage of

Table 2 | List of TLR heterodimers and homodimers, their respective pamps, ratio of binding, affinity, and cellular location

TLR Heterodimer	PAMP	Ratio of TLR Dimer to PAMP	Affinity	Location
1 and 2	Pam2	1:1	High	Cell surface
2 and 6	Pam3	1:1	High	Cell surface
3	dsRNA	1:1	High	Endosome
4 and MD2	LPS	1:2	Low	Cell surface
5	Flagellin	1:1	High	Cell surface
7	ssRNA	1:1	Low	Endosome
8	ssRNA	1:1	Low	Endosome
9	CpG	1:2	High	Endosome

kinetic control over activation, allowing agonists to be co-delivered for simultaneous TLR activation or delivered sequentially to facilitate differential activation.

Chen and colleagues demonstrated that the combination of the TLR7 agonist resiquimod (R848) and the TLR3 agonist polyinosinic: polycytidylic acid (polyI:C) induced synergistic anti-tumor immunity in a mouse model of Lewis lung cancer.[26] The authors reported that the enhanced anti-tumor effects occurred through the reprogramming of tumor-associated macrophages into an inflammatory M1 phenotype and the activation of DCs. Other studies have reported that this combination induces faster and more sustained pro-inflammatory cytokine secretion in BMDCs, revealing more nuanced kinetic effects of using multi-TLR agonists.[27] Moreover, TLR3 and TLR9 agonists have been used in combination with immune checkpoint inhibitors to further improve the efficacy of cancer vaccines.[28]

Zang et al. observed synergistic effects when a TLR7/8 agonist, imiquimod, and the TLR9 agonist CpG ODN 1826 (CpG) were combined with the Japanese encephalitis chimeric DNA vaccine.[29] The authors demonstrated an early activation of antigen-presenting cells (APCs) when the DNA vaccine was combined with the TLR agonists. Whitmore and colleagues showed that this agonist combination also induced potent anti-tumor effects in B16-F10 experimental pulmonary metastases.[30] This combination was also found to enhance antibody responses to HIV-1 vaccines in Rhesus monkeys.[31]

Orr and colleagues enhanced the efficacy of a tuberculosis vaccine called ID93, which consists of four *Mycobacterium tuberculosis* proteins, by supplementing it with GLA-SE (a TLR4 agonist) and CpG (a TLR9 agonist). The mechanism proceeded in a TRIF-independent pathway leading to an upregulation of the TH1 response. Using MPLA or GLA as a TLR4 agonist with CpG, Raman and colleagues improved the efficacy of the Leishmaniasis vaccine by similarly skewing the response towards a TH1 response, combined with increased IL-12 cytokine secretion.[32] A transcriptomic study by Lampe and colleagues in DCs revealed that this combination induced antiviral gene expression and activated the interferon regulatory factor pathway.[33]

Goff and colleagues used a synthetic TLR4 agonist, 1Z105, and a synthetic TLR7 agonist, 1V270, in combination with recombinant hemagglutinin to induce increased protection in murine influenza models.[34] A synthetic agonist targeting both TLR2 and TLR7 reactivated latent HIV-infected cells, enabling recognition and elimination by host immune cells.[35]

Other studies tested three TLR agonists together (TLR3, 4, and 8) in human monocyte-derived DCs and observed synergy. Mechanistic investigations revealed the enhanced activation of multiple signaling pathways, including NF-κB, IRF, MAPK, PI-3K, and STAT.[36] In a randomized controlled trial using the TLR3 agonist PolyI:C and the TLR4 agonist LPS, delivered with incomplete Freund's adjuvant, increased T-cell responses were observed in cancer patients.[37]

Covalently-Linked TLR Agonists

TLR agonists can be covalently linked to each other to induce synergy. However, many studies suggest that the immune responses generated are directly a result of the ligation strategy and the targeted TLRs. Ignacio and colleagues extensively reviewed the structural requirements for the chemical conjugation of TLR agonists.[38] This review discusses some of the existing scaffolds that have been used to date.

Mancini and colleagues used an α,ω-heterotelechelic PEG-based linker to synthesize a heterodimeric construct containing lipoteichoic acid (a TLR2/6 agonist) and CpG (a TLR9 agonist). The heterodimer enhanced NF-κB activation in macrophages and improved antigen cross-presentation and T-cell expansion in DCs compared to equimolar unlinked mixtures. Ryu et al. further explored the correlation between the spatial distance of the same heterodimer construct and the resulting synergy using Peg linkers of varying lengths (n = 6, 12, or 24). They observed a linker length-dependent synergy in macrophages, as measured by NF-κB activation, but not in primary cells, as assessed by increased inflammatory cytokine levels. The authors repeated similar experiments with other heterodimer pairs, combining CpG (a TLR9 agonist) with either Pam3CSK4 (a TLR2/6 agonist) or pyrimidoindole (a TLR4 agonist). Among these, the TLR4-TLR9 agonist showed the highest synergistic response when separated by Peg12 linker, indicating that an optimal distance between the two agonists was crucial for optimal immune activation. These results also demonstrate a TLR specificity in dictating the downstream immune responses.

A study using the chimeric molecule called PamadiFectin, which consists of CL307 (TLR7 agonist) covalently linked to Pam2CSK4 (a TLR2 agonist), demonstrated increased DC maturation and $CD8^+$ T cell activation. Furthermore, this new dimer induced

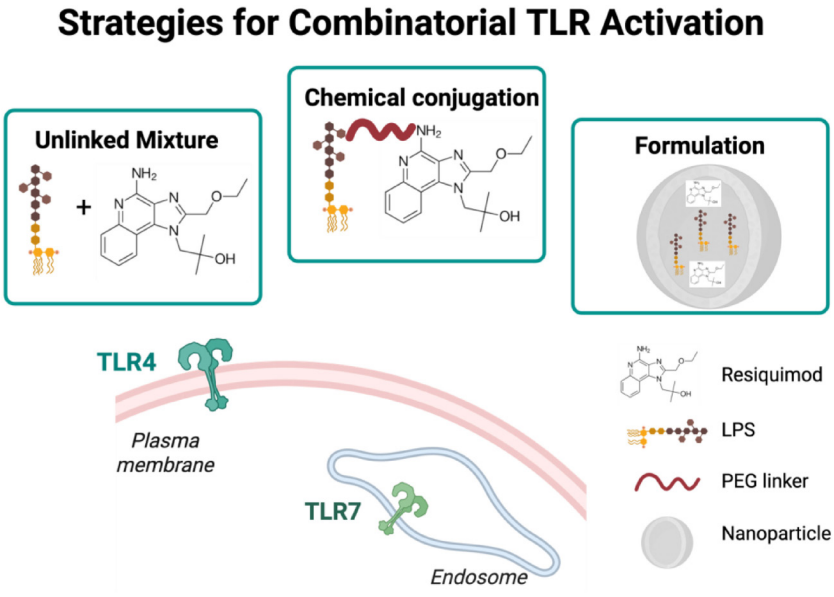

Fig 2 | Graphic representation of hypothetical dual TLR targeting using unlinked mixtures, chemical conjugation, or formulations

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higher humoral immunity compared to the unlinked mixture, demonstrating synergy in targeting a cell membrane and endosomal TLR simultaneously.[39]

Tom et al. reported the synthesis of a TLR triagonist consisting of pyrimidoindole (TLR4 agonist), CpG (TLR9 agonist), and loxoribine (TLR7 agonist) conjugated to a small molecule core. This novel agonist activated multiple immune signaling pathways, leading to increased NF-κB activation and the production of inflammatory cytokines. Furthermore, mice vaccinated with the linked TLR agonists exhibited an increase in antibody levels against the vaccinia virus compared to those vaccinated with the unlinked mixture. Albin and colleagues further demonstrated key features contributing to the type of downstream immune responses by synthesizing and evaluating five triagonist constructs targeting various TLRs.[23] Specifically, the authors noted that heterodimers conjugating a TLR9 agonist resulted in Th1 biasing and synergy, whereas those containing a TLR2/6 agonist skewed toward a TH2 response.

Nano-formulations to Deliver Multiple TLR Agonists

Delivering multiple TLR agonists using nano- and micro-particle-sized formulations presents unique advantages. Firstly, due to their size, they closely mimic natural pathogens. Secondly, the larger size permits these constructs to be endocytosed by professional APCs such as macrophages and DCs, thereby activating an adaptive response. Thirdly, particles can be formulated by employing various chemical moieties that confer conjugation strategies to present TLR agonists in distinct architectures. This text describes four types of nanoparticle (NP) scaffolds: lipid-based, protein-based, synthetic polymer-based, and carbohydrate-based.

Lee and colleagues developed a novel lipopeptide adjuvant, L-pampo™, exploiting the synergy between a combination of TLR1/2 (Pam3Csk4) and TLR3 agonists (polyI:C).[40] This novel adjuvant improved both antigen-specific antibodies and $CD4^+$ T-cell responses. The same group has demonstrated the wide-ranging applications of this adjuvant in developing effective anti-tumor therapeutics, targeting DCs and improving checkpoint blockade therapy, as well as enhancing the efficacy of vaccines, including those for COVID-19 and Herpes Zoster.[41–44] Other studies have also utilized nanostructured lipid carriers to co-deliver Pam2CSK4 (a TLR2/6 agonist) and an Imidazoquinoline derivative (a TLR7 agonist), eliciting robust protection against influenza in mice.[45]

Another study by Kuai et al. also examined the immune response generated by a combination of TLR4 and TLR9 agonists containing MPLA and CpG, respectively.[46] The agonists were encapsulated in a synthetic high-density lipoprotein nanodisc composed of biocompatible phospholipids and apolipoprotein A1 (ApoA1)-mimetic peptide. This novel construct demonstrated versatility and a broad range of activity, including enhanced humoral immunity and $CD8^+$ T cell activation-mediated anti-tumor effects.

Manna and colleagues developed supramolecular nanotherapeutics based on an amphiphilic carbohydrate polymer consisting of a covalently linked TLR agonist heterodimer targeting TLR2/6 and TLR7.[47] The nanotherapeutic was generated through the co-assembly of the heterodimer amphiphile and the amphiphilic carbohydrate polymer via noncovalent interactions. The authors demonstrate increased anti-tumor efficacy in a B16.F10 mouse melanoma model and decreased off-target cytotoxicity using the new constructs compared to an unlinked mixture.

Poly(lactic-co-glycolic acid) (PLGA)-based NPs have also been explored for delivering multiple TLR agonists that closely mimic natural pathogens. For example, NPs modified on their surface with MPLA (a TLR4 agonist) and encapsulating CpG (a TLR9 agonist) induced higher levels of inflammatory cytokines, a TH1 response, and $CD8^+$ T-cell-mediated cellular immunity in a vaccination model in mice compared to the use of single agonists. This construct is intriguing as it induces increased immunogenicity compared to PLGA NPs that are surface-modified with CpG, highlighting the critical role of ligand structural organization in inducing synergistic responses. Madan-Lala and colleagues also utilized PLGA microparticles for the combinatorial controlled delivery of multiple TLR agonists. The combinations tested included three agonists—Pam3CSK4, MPLA, and R837—agonists for TLR2, 4, and 7, respectively, encapsulated within the particles and surface-modified with CpG (a TLR9 agonist). In contrast to the previous study, a combination of surface-CpG and encapsulated MPLA induced synergy in primary DCs and *in vivo* vaccine models. This may be due to the difference in the carrier's structure and the change in targeted TLRs.

Another report conjugated Polyethylenimine (PEI) to PLGA to form NPs, which were encapsulated with either MPLA (TLR4 agonist) or resiquimod (TLR7/8 agonist), and later co-assembled with CpG (TLR9 agonist).[48] These dual antigen constructs with one embedded and one surface agonist elicited effective Th1 and IgG2a antibody-mediated responses compared to single agonist constructs. Other studies encapsulating the same combination elicited robust protection against simian immunodeficiency virus in Rhesus macaques.[49,50]

Protein NPs exhibit biocompatibility, biodegradability, and immunomodulatory potential. Ramirez and colleagues utilized an E2 protein NP platform to co-deliver the TLR5 agonist flagellin, the TLR9 agonist CpG, and the influenza hemagglutinin (H5) antigen. The authors observed robust antibody production, a balanced Th2/Th1 response, and a 100% survival rate in animals challenged with H5N1 influenza.[51]

Summary

In summary, each delivery strategy offers unique advantages. For example, using simple unlinked mixtures ensures rapid diffusion of TLR agonists to their respective receptors. However, this can be problematic due to the unwanted activation of non-specific cells and tissues,

leading to adverse effects. Conjugated TLR agonists provide better control over co-activation, as they present ligands and activate receptors simultaneously. However, not all TLR ligands can be conjugated due to issues with solubility and synthesis. Nanoformulations offer a solution by providing both cell and tissue targeting capabilities through modification of the outer membrane to recognize cell and tissue-specific signatures. However, they face challenges such as variability among different batches, longer protocols, increased costs, and time consumption (Table 3).

Future Perspectives

In Vitro Platforms to Test Synergy

Understanding the synergistic potential of combinatorial TLR agonists requires robust and scalable in vitro platforms that can accurately replicate key immune signaling events. Recent innovations in high-throughput and high-content screening technologies have enabled precise analysis of TLR-mediated responses, providing critical insights into how dual or multi-agonist strategies may lead to improved immunogenicity. Using cells instead of *in vivo* models greatly minimizes the cost of research.

Garcia-Cordero and colleagues developed a multiplexed high-throughput nano-immunoassay chip to test the synergy among pairwise TLR agonists. The authors also demonstrated that this in vitro setup translated to complex *in vivo* immunogenicity tests in mouse models. Since TLR activation results in NF-κB translocation to the nucleus, various imaging platforms can be developed to study synergy. Njikan and colleagues designed a similar setup using a high-content imaging platform, achieving a hit rate of 0.38% among the 40,000 compounds screened. Chew and colleagues recently developed high-throughput screening platforms using human primary cells, which enable a greater correlation with clinical effects.[52] Despite these developments, *in vitro* systems will need to be validated in clinical trials to replicate the complexity of human systems accurately.

AI for Predictive Modeling of TLR Agonist Efficacy

AI and machine learning algorithms can predict the immunogenic potential of TLR agonist combinations, optimizing vaccine and immunotherapy designs. By analyzing extensive datasets, AI models can identify synergistic interactions among different TLR pathways. For example, Tang and colleagues utilized data from *in vitro* high-throughput screens measuring NF-κB activation to train data-driven predictive models.[53] Through this approach, the authors generated a library of novel small-molecule immune modulators, presenting a new method for discovering synergies. The major drawback of this method is the need for high-quality in vitro datasets to train AI models, which is limited for TLR agonist combinations.

Another avenue that explores AI and machine learning for the interpretation of downstream immune effects of TLR agonist stimulation. For example, Omange et al. used this approach to analyze high-content images to identify unique cytological features in PBMCs exposed to different TLR agonists.[54]

Computational Design of Novel TLR Agonists

Computational methods facilitate the rational design of synthetic molecules that target multiple TLRs simultaneously. Utilizing information about binding affinities and receptor interactions derived from crystal structures enables the development of novel agonists with enhanced immunostimulatory properties.[55]

Notably, recent work by Adams and colleagues demonstrated the use of computational protein design to create new proteins that bind to TLR3.[56] These new proteins bound to the receptor with nanomolar affinities and induced NF-κB activation in cells. Co-receptors of TLRs can also be targeted to develop unique agonists. For example, Michaeli et al. used computational design methods to predict peptides that bind to myeloid differentiation 2 (MD-2) and cluster of differentiation 14 (CD14), two known co-receptors of TLR4.[57] However, studies need to thoroughly investigate the absence of any off-target effects contributing to unwanted cytotoxicity.

Variability in TLR Expression

TLR expression depends on age and tissue type.[58] Not all tissues express the same TLRs; therefore, the delivery route of combination agonists must consider the availability of sufficient receptors. Nano-formulations can be tailored to achieve tissue-specific delivery, thereby inducing optimal immunogenicity. Additionally, while newborns show low TLR expression, aging is associated with a dysregulated TLR expression profile.[59] Furthermore, disease conditions, such as cancers, also affect TLR expression. This can both promote cancer growth and increase host susceptibility.[60] Understanding these differences in TLR expression patterns is critical for utilizing combinatorial agonists in the treatment of various infectious diseases.

Table 3 | Comparison of various delivery strategies used to deliver multi-TLR agonists

Delivery Strategy	Advantages	Disadvantages
Unlinked mixtures	Easy, rapid diffusion, control over kinetics of activation	Poor spatial control
Covalently conjugated mixtures	Improved targeting, higher specificity, and co-delivery	Limitations of chemical conjugation due to varying chemical structures and difficulty in synthesis
Nanoformulations	Better temporal control achieved by changing the thickness of coating material, improved spatial control, and cell-targeting, efficient co-delivery	Difficulty in encapsulation based on polarity and chemical structure, batch variation, time-consuming, and expensive

Conclusion

TLRs play a crucial role in inducing a robust immune response against pathogens. Simultaneous activation of multiple TLRs mimics natural infections, thereby eliciting synergistic immune responses. This feature can be utilized to create novel adjuvants for vaccines against tumors and other infectious diseases. However, several factors govern the interaction and activation of multi-TLRs, including ligand affinity, receptor location

and expression, as well as the structural and temporal conditions necessary for activation. Given these many factors, several strategies have been employed to co-deliver multi-TLR agonists, including mixtures in solutions, covalent linking, and delivery using NPs and microparticles. Much about the mechanistic details of multi-TLR activation remains unclear due to variations among populations, tissues, and disease conditions. A better understanding of these mechanisms will help optimize the use of combinatorial strategies to develop personalized treatments for a wide variety of diseases.

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