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Strategies for designing synthetic immune agonists

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Summary

Enhancing the immune system is a validated strategy to combat infectious disease, cancer and allergy. Nevertheless, the development of immune adjuvants has been hampered by safety concerns. Agents that can stimulate the immune system often bear structural similarities with pathogen-associated molecular patterns found in bacteria or viruses and are recognized by pattern recognition receptors (PRRs). Activation of these PRRs results in the immediate release of inflammatory cytokines, up-regulation of co-stimulatory molecules, and recruitment of innate immune cells. The distribution and duration of these early inflammatory events are crucial in the development of antigen-specific adaptive immunity in the forms of antibody and/or T cells capable of searching for and destroying the infectious pathogens or cancer cells. However, systemic activation of these PRRs is often poorly tolerated. Hence, different strategies have been employed to modify or deliver immune agonists in an attempt to control the early innate receptor activation through temporal or spatial restriction. These approaches include physicochemical manipulation, covalent conjugation, formulation and conditional activation/deactivation. This review will describe recent examples of discovery and optimization of synthetic immune agonists towards clinical application.

Keywords: adjuvants; drug discovery; immuno-oncology; Toll-like receptor; vaccine.

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Introduction

Therapeutic use of immune potentiators holds great promise where the enhancement of the immune response to either foreign or endogenous antigens is the desired outcome. Beginning with the work of Jenner, vaccinology has been a proven science, and vaccines have eliminated many deadly diseases such as smallpox.[1] As vaccine developers have begun to move away from live-attenuated microorganisms to subunit vaccines, they have also stripped away many of the immune-stimulating agents naturally embedded within pathogenic bacteria or viruses, ultimately resulting in reduced vaccine efficacy. Hence, subunit prophylactic vaccines often require the addition of exogenous adjuvants.[2] Immune activation also has the potential to treat established infectious diseases. Most of the antiviral and antibacterial drugs used today target pathogens. However, in many situations, pathogens can mutate and become resistant to these drugs. Alternatively, pathogens can evade or even blunt the host immune response. Raising or restoring host immunity could be a solution to treat chronic infections that are difficult to cure.[3] Furthermore, vaccination can be extended beyond protection against infectious pathogens to priming of an immune response against self-antigens that have become transformed or malignant.[4] Over a century ago, the famous experiment by Coley demonstrated the potential of immune therapy by injecting bacteria into cancer patients.[5] Today, many investigational cancer vaccines incorporate powerful adjuvants to overcome the immune suppressive tumour microenvironment or to break self-tolerance. Lastly, the hygiene hypothesis suggests that the lack of childhood exposure to infectious microorganisms increases the susceptibility to atopic allergic disorders,

Abbreviations: BCG, bacillus Calmette–Guérin; HBV, hepatitis B virus; MPLA, monophosphoryl lipid A; PEG, polyethylene glycol; PRR, pattern recognition receptors; ssRNA, single-stranded RNA; STING, stimulator of interferon gene; Th2, T helper type 2; TLR, Toll-like receptors

because the immune system suffers from a lack of tolerance induction to the environment during early development.[6] One experimental approach to suppress the allergic T helper type 2 (Th2) response is to raise the opposing Th1 response through the appropriate type of immune potentiators. Collectively, although immune potentiators could be used in many beneficial ways to treat infectious disease, cancer and allergy, immune activation is a double-edged sword, and the development of novel immune adjuvants has been hampered historically by poor safety and tolerability. Concepts or technologies to uncouple immune-mediated efficacy from toxicity are therefore key to novel immune agonist design (Fig. 1).

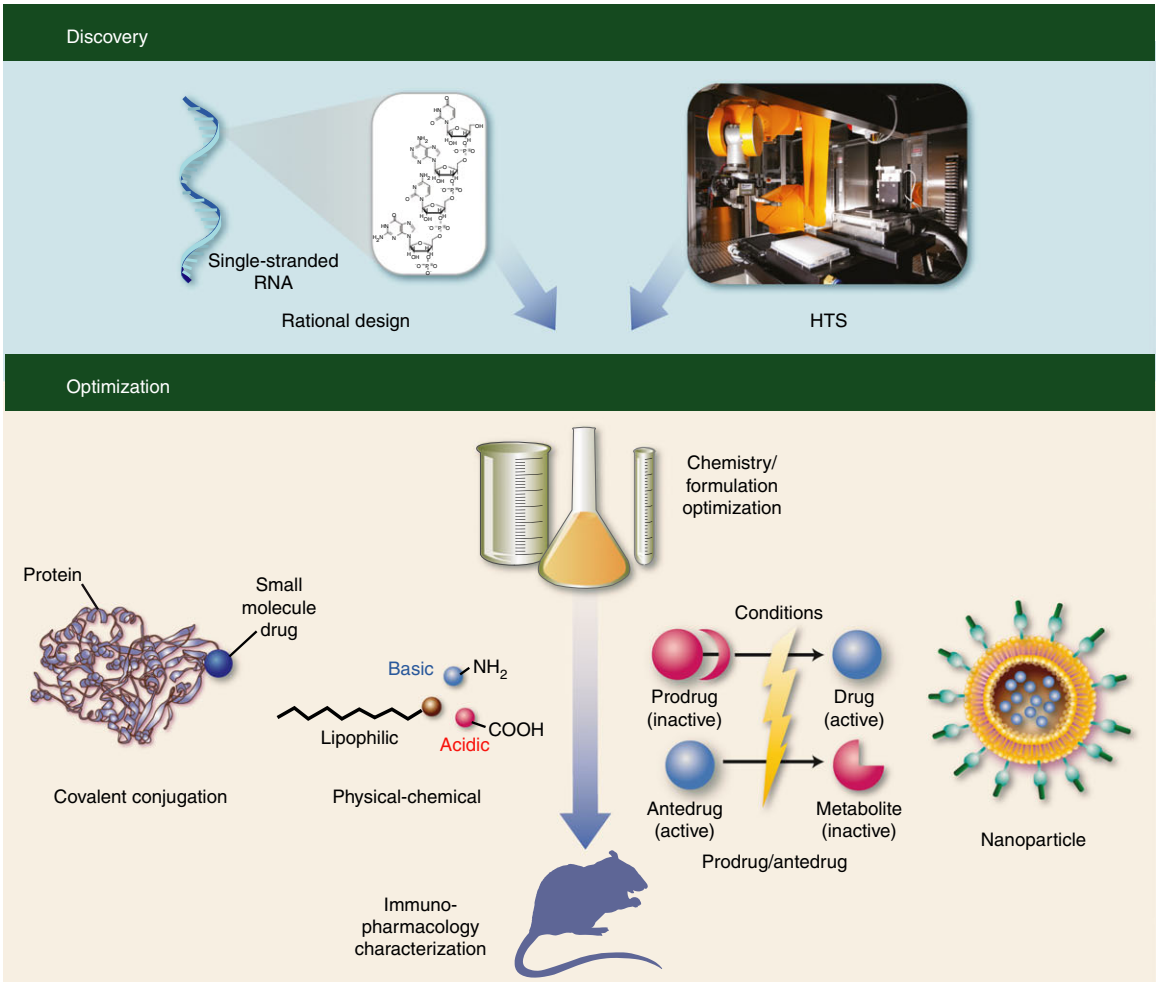
Immune agonists often structurally resemble bacterial or viral components that are recognized by pattern recognition receptors (PRR), the most studied being Toll-like receptors (TLRs).[7] TLR1/2 and TLR2/6 recognize the N-terminus of lipoproteins from Gram-positive bacteria and other microbial sources. TLR4 is stimulated by lipopolysaccharide and its derivatives, produced by Gram-negative bacteria. Bacterial flagellin is a ligand of TLR5. Of the viral recognition receptors, TLR3 senses double-stranded RNA, whereas TLR7 and TLR8 sense single-stranded RNA (ssRNA). TLR9 is stimulated by unmethylated CpG motifs commonly found in bacterial DNA. Besides TLRs, nucleotide-binding oligomerization domain-like receptors are stimulated by bacterial products.[8] Retinoic acid-inducible gene-1-like receptors detect the presence of viral infection.[9] C-type lectin receptors recognize a variety of carbohydrate derivatives.[10] The most recently discovered PRR, stimulator of interferon gene (STING), is a sensor of cytosolic DNA.[11]

Although all PRR ligands can be derived from microbial sources, many of these natural product derivatives have undefined structures (e.g. complex glycolipids) that are often polymeric, which make manufacturing on a commercial scale a daunting task. The mechanism of action is often complicated, and these natural products exhibit poor physicochemical properties, which presents formulation challenges compared with modern small-molecule drug standards. Progressively, with a better understanding of innate receptor biology, researchers have explored different ways to simplify or synthesize pathogen-associated molecular pattern mimetics using chemistry to improve pharmacological properties. New adjuvant chemotypes often bear structural resemblance to the natural ligands, but are derivatized in ways that increase and/or maintain the adjuvant potency, while reducing the structural complexity for ease of manufacture and improving other important characteristics.

Most modern small-molecule drugs have gone through iterative rounds of optimization, often with the goal of increasing systemic bioavailability and extending *in vivo* half-life for maximum therapeutic effects. Immune adjuvants, however, follow different design principles. Adjuvants function by stimulating antigen-presenting cells to more effectively uptake and present antigens through inflammatory cytokines and co-stimulatory molecules. This enhanced process leads to better adaptive humoral and cellular responses. Without the intended antigen(s) in proximity, adjuvants induce non-productive immune activation, or wasted inflammation, which contributes little to adaptive immunity. Literature reports have demonstrated that systemic distribution of vaccine adjuvants is not required to elicit antigen-specific immune responses.[12,13] Moreover, repeated systemic administration of TLR agonists has been shown to blunt the innate response, possibly through receptor desensitization or other mechanisms of immune tolerance induction.[14,15] Hence, for better efficacy and improved tolerability, a key attribute of adjuvant distribution should be co-localization with the antigen(s) it serves to boost immunity against. Systemic distribution and systemic immune activation are not needed nor desired to induce an antigen-specific adaptive immune response.

Chemistry functionalization can be used to enhance the biological activities and drug-like properties of small molecules. Strategies of adjuvant optimization often involve tuning selectivity and reducing off-target activity, simplifying structural complexity, increasing stability, enhancing injection site retention, improving antigen/adjuvant co-delivery, or engineering conditional activation/

Figure 1. Discovery, optimization and application of synthetic immune agonists. Discovery of novel immune agonists can come from rational design based on pathogen-associated molecular patterns or from un-biased high-throughput screening of diverse compound libraries. Chemistry/formulation optimization can follow a number of strategies with the common goal of eliciting antigen-specific immune response. Co-delivery of antigen and adjuvant can be achieved through covalent conjugation or nanoparticle formulation. The physical–chemical properties of synthetic immune agonists can be engineered to allow preferential tissue distribution. Conditional activation/deactivation by designed prodrug/antedrug attempts to restrict the immune activation within certain tissues. Optimized agonists are then characterized in various immuno-pharmacological animal models of their intended clinical applications. Different routes of administration are required for local delivery to afford the greatest benefit-to-risk ratio (therapeutic index). Vaccine adjuvants are administered parenterally, either through subcutaneous or intramuscular injection, together with the antigens. Autologous vaccination injects the immune agonists directly into the tumour to generate an immune response against tumour antigens *in situ*. Topical applications are used to treat dermatological diseases. Intranasal administration and inhalation of immune agonists are investigated for their ability to suppress allergy or asthma. Oral TLR7 agonists have been explored for treatment of hepatitis C virus and hepatitis B virus. Intravesical delivery of bacillus Calmette–Guèrin (BCG) directly into the bladder is currently the standard of care for non-invasive bladder cancer.



Parenteral, intratumoral* (vaccine, cancer)		
Hitonol	TLR3	Oncovir
Ampligen	TLR3	Hemispherx
MPLA	TLR4	GSK
G100*	TLR4	Immune design
Entolimod	TLR5	Cleveland biolabs
3M-052*	TLR7/8	3M/Medimmune
VTX-2337	TLR8	VentiRx
1018 ISS	TLR9	Dynavax
MGN1703	TLR9	Mologen
PF-3512676*	TLR9	Pfizer
SD-101*	TLR9	Dynavax
IMO-2125*	TLR9	Idera
CMP-001*	TLR9	Checkmate pharma

Intravesical (urology)		
BCG	TLR2/4	Merck
TMX-101	TLR7	Telormedix

Topical (dermatology, cancer)		
Aldara	TLR7	3M/Graceway pharma
R848 gel	TLR7/8	3M

Intranasal/inhaled (allergy, asthma)		
AZD8848	TLR7	Astra-Zeneca
GSK2245035	TLR7	GSK

Oral (HCV, HBV, HIV)		
R848	TLR7/8	3M
RG7795	TLR7	Roche
PF-4878691	TLR7	Pfizer
GS-9620	TLR7	Gilead

deactivation. Cumulative knowledge gained from systematic adjuvant optimization has provided the guiding principles for designing safe and effective adjuvants.[16–20] This review will illustrate strategies of synthetic immune agonist discovery and optimization toward clinical application.

Discovery from rational design (inspired by nature)

Among the known innate immune receptors, TLR7 was the first receptor reported to be activated by small molecules; hence, it has been the target for which most small-molecule agonists have been generated and researched. TLR7 recognizes viral ssRNA, which consists of repeating units of ribonucleotides.[21,22] However, even before the discovery of TLR7, small heterocyclic molecules bearing nucleoside-like structure were reported to exhibit therapeutic effects through immune potentiation. As early as the 1970s, Nichol *et al.*[23] reported the interferon-inducing activity of a substituted pyrimidine, a scaffold shared by nucleic acid bases cytosine and thymidine. During the 1980s, Goodman and Weigle[24] first reported the lymphocyte activation activity of a purine nucleoside analogue. In the late 1980s, Bernstein *et al.* and Chen *et al.*[25,26] reported antiviral activity of an imidazoquinoline named R837/S26308 (later named imiquimod) in guinea-pig models of herpes simplex virus and cytomegalovirus. Building on the earlier findings, Hirota *et al.*[27] described the structural activity relationship of a series of non-nucleoside adenine analogues as potent interferon inducers without knowing the target. It was not until 2002 that Hemmi *et al.*[28] identified the target of imiquimod to be TLR7. A year later, Lee *et al.*[29] demonstrated that purine nucleoside analogues also function through TLR7. Relative to complex natural products and biologics, small synthetic molecules can be more easily made into defined drug candidates using controlled and reproducible manufacturing processes. Hence, the discovery that low-molecular-weight agonists can stimulate TLR7 in a manner comparable to the natural ligand, ssRNA, prompted biotech and pharmaceutical companies to search for novel and proprietary chemical space. Expanding on the earlier chemotypes, a series of deazapurine,[30] pyrimidine,[31] pteridinone,[32] benzonaphthyridine,[33] and other scaffolds have been disclosed in publications and patents.[34] It is of interest to note that most of these TLR7 chemotypes share a common 2-aminopyridine or 2-aminopyrimidine core, which could be a critical nucleic acid recognition motif for the receptor. Although the structure of TLR7 has yet to be solved, researchers have built homology models based on the X-ray crystal structure of the TLR8 ectodomain in an attempt to rationalize the observed structure activity relationship and guide future medicinal chemistry optimization.[32]

Similar to TLR7, TLR8 is also an endosomal TLR that recognizes ssRNA. The main differences between the two RNA recognition receptors lie in their expression and functional activity across different species. Whereas TLR7 is predominantly expressed in plasmacytoid dendritic cells, TLR8 is expressed in myeloid dendritic cells, monocytes and macrophages.[35,36] Interestingly, rodents lack a functional TLR8, making it challenging to characterize TLR8 agonists *in vivo*.[37,38] CL075 is an imidazoquinoline TLR7/8 agonist reported to have more TLR8-biased activity.[39] Extending from the imidazoquinoline series, Kokatla *et al.*[40] identified a series of furoquinolines that displays much improved selectivity over TLR7. Recently, the X-ray co-crystal structure of TLR8 has been solved with a number of small-molecule ligands.[41,42] Guided by this information, structure-based ligand design led to the identification of several additional TLR8 agonists.[43,44]

Bacterial lipoproteins and lipopeptides are known to stimulate the innate immune system through TLR2.[45,46] Even before the discovery of TLR2, *N*-acyl-*S*-diacylglyceryl cysteine motifs were known simply as macrophage-activating lipopeptides.[47] X-ray crystallography revealed that the number of acyl chains determines selectivity between TLR1/2 versus TLR2/6 heterodimerization. Triacylated lipopeptides activate TLR1/2 by insertion of the *N*-acyl chain to TLR1 and the remaining two glyceryl acyl chains to TLR2.[48] In contrast, di-acylated lipopeptides, with the N-acyl chain missing, activate TLR2/6.[49] Detailed structure activity relationship studies of the cysteine-glycerol core showed the importance of stereochemistry, the thioether bridge, and ester connections.[50] The peptide region can tolerate more functional group manipulation. Many synthetic lipopeptides have introduced polyethylene glycol (PEG) or sugar moieties at the peptide region with the aim of improving solubility and/or amphiphilic properties of these otherwise poorly soluble and lipophilic molecules.[51–53] Further simplifying the structure, a series of monoacyl lipopeptides was discovered to be human-specific TLR2 agonists.[54,55] Another simplified class of lipoamino acid agonist was identified by maintaining the minimal structure requirement for TLR2 activity.[56]

The natural ligand of TLR4 is bacterial lipopolysaccharide.[57] X-ray crystal structure studies have shown that the recognition element lies in the lipid A portion through the adaptor protein MD2.[58] Mono-phosphoryl lipid A (MPLA) is a TLR4 agonist produced from alkaline hydrolysis of salmonella-derived lipopolysaccharide.[59] The structure of MPLA is not synthetically defined, as the product contains a mixture of derivatives with five and six alkyl chains. It is clinically approved as an adjuvant in hepatitis B virus (HBV) and human papillomavirus vaccines. Apart from the semi-synthetic MPLA, fully synthetic derivatives of lipid A have been reported including glucopyranosyl lipid A,[60] aminoalkyl glucosaminide 4-phosphate[61] and E6020.[62]

Discovery from high throughput screening

With the advent of new screening technologies with the ability to assay large collections of small-molecule compound libraries in miniaturized cellular assays, both industrial and academic institutions have turned to high-throughput screening as a modern way of identifying novel small-molecule immune potentiators. VentiRx has reported a series of benzoazepine TLR8 agonists identified from screening.[63] The lead candidate, VTX-2337, has been shown to activate natural killer cells and enhance antibody-dependent cell-mediated cytotoxicity in human cells *in vitro*, and is being developed for cancer indications.[64] Another benzoazepine derivative, VTX-294, was reported to activate newborn and adult leucocytes better than other TLR7/8 agonists R848 and CL075.[65] Other human-specific small-molecule agonists discovered from high-throughput screening have been reported for TLR2,[66] TLR4[67,68] and STING.[69] Interestingly, given that these screenings were conducted in human cells, many of these agonists were reported to exhibit significantly reduced activity in mouse cells.

Physicochemical manipulation

Most low-molecular-weight drugs are designed with the intent for oral bioavailability and systemic delivery. In contrast, immune adjuvants should be co-localized to the antigen(s) against which the immune response is intended. Restricting the spatial distribution of adjuvants prevents systemic activation of peripheral immune cells, and thereby minimizes inflammatory cytokine production that is not contributing to the antigen-specific immune response. As vaccines are often given parenterally, one way to provide localized immune activation is through increasing local injection site retention. This could be achieved by manipulating the physicochemical properties of the small molecule adjuvant. Towards this end, Smirnov *et al.*[12] installed long lipophilic alkyl moiety for slow dissemination from the site of application. Upon subcutaneous injection, 3M-052 drives a strong Th1 response to haemagglutinin and serum neutralization of viable H1N1 virus in the absence of circulating tumour necrosis factor-α or the induction of Th1 cytokines. Intratumoral administration of 3M-052 in the B6.F10 melanoma model generated systemic antitumour immunity and suppressed both injected and non-injected (distal) tumour growth.[70] Similarly, Chan *et al.*[71,72] described the synthesis and characterization of adenine TLR7 agonists modified with PEG and/or phospholipid for improved pharmacokinetics and biodistribution. Installation of a phospholipid onto the benzylic region of 8-oxo-adenine increased *in vitro* potency 100-fold over the unmodified TLR7 agonist and induced both Th1 and Th2 antigen-specific immune responses in an ovalbumin model.[71] The phospholipid–adenine conjugate was further demonstrated to reduce cancer growth in a B16c-ovalbumin melanoma model via intralesional administration.[73] Shukla *et al.*[74] designed TLR7-agonistic dendrimers with three to six units of the active imidazoquinoline pharmacophore. These high-molecular-weight dendrimers retain potent TLR7 activity and were shown to induce high-affinity antibodies to bovine α-lactalbumin. Taken together, there are multiple ways in which synthetic immune agonists can be localized through physical chemical parameters, such as increasing molecular weight and lipophilicity (logP), or reducing polarity (polar surface area) and solubility.

Covalent conjugation

In order to elicit a more efficient and specific immune response, an effective vaccine delivery system would function to target the immune agonist (adjuvant) and antigen to the same antigen-presenting cells. To this end, researchers have explored various approaches to covalently conjugate adjuvant and antigen. Wille-Reece *et al.*[75,76] have demonstrated that covalent linking of an imidazoquinoline TLR7/8 agonist to HIV Gag protein dramatically enhanced the magnitude and altered the quality of the Th1 response, compared with animals co-immunized with HIV Gag protein (non-conjugated) and the TLR7/8 agonist or CpG-oligodeoxynucleotide. Follow-up mechanistic studies revealed that the TLR agonist–antigen conjugate elicits $CD8^+$ T-cell responses based not on the capacity to induce dendritic cell maturation or antigen persistence and uptake, but on the engagement of dendritic cell cross-presentation pathways.[77] Vecchi *et al.*[78] described the conjugation of a TLR7 agonist to *Streptococcus pneumoniae* antigen, which resulted in dose-sparing of both antigen and adjuvant, and it also protected mice from a lethal challenge. No adjuvant effect was observed when equimolar unconjugated TLR7 agonist was co-administered together with the non-conjugated antigen. Other examples of TLR agonist conjugation to peptide antigens have been extensively reviewed.[79]

In addition to vaccine antigens, conjugation of small-molecule immune agonists to other macromolecules such as proteins, carbohydrates or antibodies can be used to achieve special properties. Wu *et al.*[80] showed that covalent attachment of an adenine TLR7 agonist to mouse serum albumin can increase local immune activation and reduce systemic inflammation. When administered into mouse lung *in vivo*, the TLR7 agonist–mouse serum albumin conjugate induced 10-fold higher local release of cytokines relative to the unconjugated TLR7 agonist. The functional efficacy of the conjugate was demonstrated in an anthrax and flu challenge model to delay mortality. Leveraging non-covalent affinity, Liu *et al.*[81] modified a TLR9-activating CpG-oligonucleotide with lipid moieties

capable of physical binding to albumin. Hitch-hiking on endogenous serum albumin, lipid-modified CpG effectively accumulated in lymph nodes, which resulted in better T-cell priming, enhanced antitumour efficacy in a B16F10 tumor model, and reduced systemic toxicity. Shinchi *et al.*[82] conjugated an adenine TLR7 agonist onto polysaccharides to improve aqueous solubility. The resulting conjugate appeared to be a more potent adjuvant, and the authors hypothesized that this was due to the enhanced uptake by the antigen-presenting cells. Lastly, to mimic the combination effect of multiple types of TLR agonists found naturally in microorganisms, Esser-Kahn and co-workers have conjugated agonists of TLR2 and TLR9, and also agonists of TLR4, TLR7 and TLR9, respectively. These spatially defined di- and tri-agonists were shown to exhibit higher activity synergistically over the unconjugated mixtures.[83,84]

Delivery of small-molecule drug via antibodies through covalent conjugation is a validated technology, as several antibody–drug conjugates have been approved for clinical treatment of cancer.[85] Extending this concept, several groups have attempted to deliver immune agonists by conjugation to antibodies targeting tumour cells. Sharma *et al.*[86] conjugated CpG-oligodeoxynucleotide (TLR9 agonist) to anti-Her-2/neu monoclonal antibody and demonstrated that the conjugate can bind to Her-2/neu$^+$ tumours, activate dendritic cells, and induce antitumour responses. Li *et al.*[87] described the conjugation of CpG to clinically approved monoclonal antibodies rituximab and trastuzumab. Recently, Gadd *et al.*[88] have extended the antibody conjugation approach to TLR7 agonists as the payload. Instead of targeting tumour cells, Kreutz *et al.*[89] reported an antibody–antigen–adjuvant conjugate designed to target DEC205$^+$ dendritic cells. The antibody–antigen–adjuvant triple conjugate was demonstrated to be superior to the antibody-free antigen–adjuvant double conjugate in priming of cytotoxic T-lymphocyte responses and efficiently induced anti-tumour immunity in a B16 model, although the authors had noted possible non-specific delivery to cells that are independent of the DEC205.

Formulation-assisted delivery

Although covalent conjugation and increased lipophilicity are strategies that have led to good tool adjuvants through localized immune activation, there are practical considerations for commercial development. Covalent attachment of immune agonist requires careful control of conjugation chemistry to afford homogeneous protein production. Each covalently modified antigen is a new biological entity that requires separate manufacturing and characterization protocols. Co-administering a vaccine antigen with a locally retained adjuvant offers the advantage of stockpiling one adjuvant formulation to be used with multiple vaccines. However, highly lipophilic and poorly soluble adjuvants often present formulation challenges, resulting in inconsistent dosing and prolonged residence time at the injection site, sometimes lasting longer than the antigens.

To better understand the ideal properties of immune adjuvant, Wu *et al.*[13] investigated the minimal essential temporal and spatial distribution required for effective adjuvanticity using structurally similar TLR7 agonists with differential physicochemical properties. Gene expression and cytokine profiling revealed that most of the immediate inflammatory activity is needed only at the injection site, and that increased inflammation in the serum does not necessarily contribute to better adjuvanticity. To address the poor developability of insoluble adjuvants, the authors subsequently designed soluble analogues that can be adsorbed onto alum via phosphonate/$Al(OH)_3$ ligand exchange. Alum has been used in human vaccines for decades.[90] It is thought that one attribute linked to its adjuvanticity is increased antigen deposition, via non-covalent adsorption. This is argued to be mediated through electrostatic, hydrophobic and ligand exchange interactions.[91] As alum behaves like a particulate at the injection site, alum adsorption of adjuvant and antigen results in a highly co-localized vaccine formulation with good development potential. Once dissociated from alum, these soluble TLR7-phosphonates are readily eliminated from the injected muscle and undergo rapid systemic clearance, resulting in minimal wasted inflammation. TLR7/alum adjuvant formulations were tested to effectively enhance a vaccine against *Neisseria meningitidis* serotype B, increasing both the depth and breadth of serum bactericidal antibody coverage against 17 *Neisseria meningitidis* serotype B strains. Furthermore, the authors demonstrated that the modification of TLR7 agonists with phosphonate to afford alum adsorption was a generalizable approach to enhance vaccine adjuvanticity in several other TLR7 chemotypes.

Polymer-based encapsulation has also been extensively investigated for adjuvant delivery.[92,93] Kasturi *et al.*[94] demonstrated that immunization of mice with poly(lactic-co-glycolic acid) nanoparticles containing antigens plus TLR7 and TLR4 ligands induced synergistic increases in antigen-specific, neutralizing antibodies. Moreover, immunization protected completely against lethal avian and swine influenza virus strains in mice, and induced robust immunity against pandemic H1N1 influenza in rhesus macaques. Ilyinskii *et al.*[95] demonstrated that co-delivery of an antigen with a TLR7/8 or TLR9 agonist in synthetic vaccine particles resulted in a strong augmentation of humoral and cellular immune responses with minimal systemic production of inflammatory cytokines. Dranoff, Mooney and co-workers have reported the incorporation of granulocyte–macrophage colony-stimulating factor (a dendritic cell enhancement factor) and

CpG (a dendritic cell activating factor) in biomaterials based on poly(lactic-co-glycolic acid),[96] mesoporous silica rods[97] and cryogel.[98] The goal of this approach is to induce a coordinated regulation of a dendritic cell network to enhance host immunity against added tumour lysate or *in situ* tumour antigens. DeMuth *et al.*[99] generated microneedle arrays coated with biodegradable cationic poly(β-amino ester) and negatively charged interbilayer-cross-linked multilamellar lipid vesicles. These interbilayer-cross-linked multilamellar lipid vesicles were loaded with protein antigen and MPLA, and the vaccination promoted a robust humoral response compared with the soluble components. Hanson *et al.*[100] demonstrated that encapsulation of STING agonist cdGMP using PEGylated lipid nanoparticle can block systemic dissemination and enhance draining lymph node accumulation, leading to increased CD8 T-cell response and antitumour immunity. Lynn *et al.*[101] investigated the physicochemical properties of polymer-linked TLR7/8 agonists by varying different linkers with agonist densities. Interestingly, improved local retention is necessary but not sufficient for enhancing T-cell immunity, as low-density unimolecular polymer coils showed reduced immunogenicity compared with high-density submicron polymer particles.

Conditional activation through prodrug and antedrug

Chemical modification of TLR agonists can be used to elicit tissue-specific immune activation through controlled drug cleavage. Fletcher *et al.*[102] designed masked oral prodrugs of TLR7 agonist that can bypass immune stimulation in the gastrointestinal tissues, thereby reducing gastrointestinal intolerances. Fosdick *et al.*[103] showed that oral administration of a pteridinone agonist (GS-9620) with high first-pass hepatic clearance induced more interferon than intravenous administration, while achieving similar systemic exposure; therefore, the majority of interferon is generated pre-systemically from the gut-associated lymphoid tissue. At low doses, GS-9620 activates interferon-stimulated genes without inducing systemic interferon and related adverse effects, providing a potential therapeutic window for inducing an anti-HBV immune response. Recently, Ryu *et al.*[104] have reported an imidazoquinoline TLR7 agonist prodrug that can be activated by light. Light-activated TLR7 agonists can be used in combination with radiation therapy, where localized irradiation of tumour can kill the cancer cells and release cancer antigens, while simultaneously activating the TLR7 prodrug for enhanced antigen uptake and presentation.

Although prodrugs are activated under certain biological conditions, antedrugs are deactivated to limit the activity within certain tissues. Administration of low-dose TLR7 agonist to the upper airway has the potential to treat various allergic diseases by skewing the immune microenvironment from Th2 to Th1.[105] To prevent undesired systemic activity, Kurimoto *et al.*[106] applied the antedrug concept to an adenine TLR7 agonist to restrict innate immune activation in the airway. The TLR7 antedrug is designed to be metabolically deactivated by plasma esterase to avoid systemic spillover. Even with transient activity in the lung, the adenine antedrug was demonstrated to effectively inhibit allergen-induced airway inflammation without inducing systemic cytokines.[107]

Clinical application

Though many potential utilities have been demonstrated in pre-clinical models, a key clinical translation hurdle for all immune adjuvants is safety. Successful clinical applications for TLR agonists have been so far limited to local delivery. R837 (imiquimod) is currently the only approved TLR7 agonist for use in humans to topically treat basal cell carcinoma.[108] Despite being effective against these skin lesions, there are significant adverse effects associated with excessive inflammation at the treated sites, which is sometimes accompanied with fever and flu-like symptoms.[109] We now know that R837 acts, at least in part, through activation of TLR7. But because it was developed before knowledge of the molecular target, it is likely that the drug was identified solely based on phenotypic activities without fully understanding the selectivity against a broader panel of proteins and receptors. Many reports have described R837 having TLR7-independent effects, which could contribute either to the efficacy and/or adverse effects.[110–112] Recently, a non-imidazoquinoline-based TLR7 agonist was reported to be equally effective in a guinea-pig herpes simplex virus model through intravaginal administration, but without the side-effects of weight loss and fever.[113] This raises the possibility that perhaps not all of the TLR7-independent effects are required for R837's efficacy. In additional to dermatological diseases, topical R848 (resiquimod) gel is also being investigated as cancer vaccine adjuvant.[114]

Parenteral TLR agonists are administered as single agents (subcutaneous), together with vaccine antigens (intramuscular), or more recently, directly into the tumour where tumour-associated antigens reside (intratumoral). AS04 is an adjuvant that contains the TLR4 agonist MPLA and alum.[115] It is approved for intramuscular injection as adjuvant in human HBV and human papillomavirus vaccines. Bacillus Calmette–Guérin (BCG) is a live-attenuated bacteria used for the treatment of non-invasive bladder cancer.[116] Its mechanism of action has been attributed to TLR2/4 activation. TMX-101 is a soluble formulation of R837 for intravesical delivery directly into the bladder.[117] In addition to being synthetic, R837 also has the advantage of being non-infectious compared

to BCG. TLR3 agonists Ampligen™ and Hiltonol™ have been used for the treatment of chronic fatigue syndrome and various types of cancer, as well as vaccine adjuvants.[118–120] The TLR5 agonist entolimod has been implicated in radiation countermeasures and cancer therapy.[121,122] A number of TLR9 agonists have been investigated as adjuvants for human vaccines, the most advanced being 1018 ISS in HBV vaccine.[123] More recently, *in situ* vaccination through intratumoral injection of TLR9 agonist (PF-3512676) has been demonstrated to induce systemic anti-tumour immunity in murine models and in patients with B-cell lymphomas.[124,125] Interestingly, in other trials the same CpG molecule given systemically through subcutaneous administration reached dose-limiting toxic levels before any therapeutic benefits were observed.[126,127] The critical abscopal response, where intratumoral administration diminishes tumour growth at both treated and non-treated sites, has also been observed with TLR7/8 and STING agonists in murine models.[70,128] Hence, several companies are currently exploring intratumoral injection of TLR4 (G100), TLR7/8 (3M-052 aka MEDI9197), or TLR9 agonists (SD-101, IMO-2125, CMP-001) in clinical trials as single agents or in combination with checkpoint inhibitors. VTRX-2337 is a TLR8 agonist also being investigated for cancer treatment in combination with chemotherapy.[129]

With regard to infectious diseases, oral TLR7 agonists such as R848, ANA773 (now RG-7795) and PF-4878691 have been explored for treatment of hepatitis C virus.[130–132] All of these investigational drugs showed adverse effects with symptoms reminiscent of interferon induction. Low doses of the TLR7 agonist GS-9620 are currently being investigated for treatment of chronic HBV,[133] which has been shown to be efficacious in the woodchuck hepatitis viral model and in HBV-infected chimps.[134,135] GS-9620 has also shown promise in reversing HIV latency.[136] In addition, intranasal TLR agonists have been investigated for allergen immunotherapy. In a phase IIa trial, AZD8848 (TLR7 antedrug) dosed intranasally was associated with sustained reduction in allergen responsiveness, although it also produced interferon-related effects.[137] AZD8848 was also tested in humans for tolerability via inhalation.[138] GSK2245035 is yet another intranasal TLR7 agonist being investigated to treat respiratory diseases.[139,140]

Conclusion

Stimulation of innate immune receptors has been implicated in the treatment of many diseases. Even before the discovery of TLRs, there were numerous reports of synthetic molecules capable of inducing cytokines and activating lymphocytes. With better understanding of the TLR structure and biology, discoveries of novel synthetic adjuvants have come from rational design or high-throughput screening. Optimization of adjuvants has aimed at increasing potency and reducing structural complexity, but most importantly, improving safety and tolerability. Strategies such as manipulation of physicochemical properties, conjugation to macromolecules, formulation-assisted delivery, and prodrug/antedrug all serve to localize the immune activation to the intended antigen(s). So far, clinical applications of immune agonists have been mainly limited to local delivery to minimize immune-related toxicities. Non-antigen-specific, systemic activity is not desired for adjuvanticity and should be minimized to avoid wasted inflammation. The ability to tune the properties of synthetic agonists through chemistry or formulation may allow broader clinical utilities of these immune agonists to benefit more patients as we understand more about them.

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Disclosures

None.

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