Review

Bioconjugation Approaches to Produce Subunit Vaccines Composed of Protein or Peptide Antigens and Covalently Attached Toll-Like Receptor Ligands

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Bioconjugation Approaches to Produce Subunit Vaccines Composed of Protein or Peptide Antigens and Covalently Attached Toll-Like Receptor Ligands

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Abstract
Traditional vaccines derived from attenuated or inactivated pathogens are effective at inducing antibody-based protective immune responses, but tend to be highly reactogenic, causing notable adverse effects. Vaccines with superior safety profiles can be produced by subunit approaches, utilizing molecularly defined antigens (e.g. proteins and polysaccharides). These antigens, however, often elicit poor immunological responses, necessitating the use of adjuvants. Immunostimulatory adjuvants have the capacity to activate antigen presenting cells directly through specific receptors (e.g. Toll-like receptors (TLRs)), resulting in enhanced presentation of antigens as well as secretion of proinflammatory chemokines and cytokines. Consequently, innate immune responses are amplified and adaptive immunity is generated. Recently, site-specific conjugation of such immunostimulatory adjuvants (e.g. TLR ligands) onto defined antigens has shown superior efficacy over unconjugated mixtures, suggesting that the development of chemically-characterized immunostimulatory adjuvants and optimized approaches for their conjugation with antigens may provide a better opportunity for the development of potent, novel vaccines. This review briefly summarizes various TLR agonists utilized as immunostimulatory adjuvants and focuses on the development of techniques (e.g. recombinant; synthetic, and semisynthetic) for generating adjuvant-antigen fusion vaccines incorporating peptide or protein antigens.

INTRODUCTION

Vaccination is one of the most important measures for the prevention of infectious diseases. Immunization with pathogen-derived antigens can elicit protection against infection caused by such pathogens through inducing antigen-specific immune responses. Several different vaccine approaches have been developed to date. Of these, the subunit vaccine approach, where a molecularly characterized component (e.g. a protein or polysaccharide) is used as an antigen, tends to exhibit a better safety profile than traditional whole organism vaccine approaches.[1] By incorporating defined pathogen components, subunit vaccines are able to generate defined antigen-specific responses. Furthermore, subunit vaccines can be: produced with minimal batch-to-batch variation in good accordance with regulatory requirements; designed to incorporate unnatural components that cannot be achieved by purification from natural sources; freeze dried for the purpose of stability and simplifying storage and transportation logistics; and can be used where pathogens are unable to be grown in culture.[2] A broad range of strategies have been used to develop subunit vaccines, including recombinant or synthesized proteins and peptides, toxoids, polysaccharides and polysaccharide-carrier protein conjugates.[2]

With the development of recombinant DNA technology, it is convenient to produce recombinant protein or peptide antigens in a highly-purified form. But, subunit vaccines, involving such antigens, are normally poorly immunogenic and cannot elicit potent immune

responses to afford protection on their own.[3] Adjuvants are therefore needed to enhance the immune response of such poorly immunogenic vaccine antigens. Adjuvants are substances used in combination with a specific antigen, which together produce a more robust immune response than the antigen alone.[4] Currently only a limited number of adjuvants have been licensed for human use, including alum salts, oil-in-water emulsions (MF59 and AS03), virosomes and AS04 (which consists of alum and monophosphoryl lipid A (MPLA)).[5-8] Although alum is the oldest adjuvant, it is still commonly used in licensed vaccines due to its good safety record. Aluminum containing adjuvants can induce T_H2 humoral and $CD4^+$ T cell responses, but in general do not stimulate cytotoxic T lymphocyte (CTL) responses.[9] Thus, the use of alum is inappropriate where cytotoxic T lymphocytes are required for vaccine success (e.g. therapeutic vaccines against cancers). Hence there is an urgent need to develop new adjuvants that can enhance and shape vaccine-induced responses. Immunopotentiating adjuvants, represented by MPLA, are under intense investigation since these types of adjuvants can activate the innate immune system via pattern recognition receptors (PRR) which subsequently lead to an adaptive immune response.[8] Dendritic cells (DCs), as TLR expressing cells, play a critical role in mediating adaptive immunity. Immunopotentiating adjuvants, such as TLR agonists, are thought to target DCs directly, thereby directing the balance between humoral and cell-mediated immunity associated with acquired immune responses.[8] From this perspective, the potential of TLR agonists as adjuvants for the next generation of vaccines should be explored. Meanwhile, it should be emphasized that any safety concerns or unacceptable levels of intolerability caused by adjuvant candidates are not acceptable.

Immunopotentiating adjuvants can be simultaneously delivered with antigens to improve vaccine efficacy. Codelivery of antigens and TLR agonists to antigen presenting cells (APCs) ensures the colocalization of both molecules to the same endosome or phagosome, within the same APC, thereby enhancing the antigen presentation and processing efficiency.[3] A number of strategies including covalent conjugation, encapsulation and entrapping antigens in lipid-based vesicles have been developed to fulfill the goal of codelivery.[10-13] Among these strategies, conjugation has received a great deal of attention due to the ability to produce molecularly defined conjugates in a controlled manner. Numerous (bio)chemical strategies have been developed to covalently attach adjuvant molecules onto antigen peptides or proteins, most of which can be summarized into three categories: synthetic, recombinant fusion proteins, and semisynthetic approaches.[12,14-19] The selection of one of these approaches needs to be rationally chosen based on the properties of the antigen and adjuvant. For example, chemical synthesis offers a cost-effective and feasible way to produce short peptides and small proteins and offers significant opportunities to engineer in non-natural components, while the routine synthesis of large peptides and proteins are less accessible due to synthetic difficulties. On the contrary, recombinant approaches are normally selected for the production of large protein molecules, but not peptides or small proteins, since peptides tend to be degraded during their expression in host cells.[20] Further, it is important for many protein antigens that their native 3-dimentional structure is maintained in order to elicit protective immune responses. In such cases, the application of synthetic approaches is limited to sequences that can be readily refolded. Further, TLR-based adjuvants comprise various types of molecules (e.g. lipo-peptides/proteins/polysaccharides, nucleic acids and proteins),[18,21-26]

with different approaches required to produce each adjuvant. Finally, semisynthetic approaches combine the advantages of both synthetic and recombinant approaches, and hence can be used to produce conjugate vaccines composed of folded protein antigens and synthetic TLR agonists. In addition, limited ability of some TLR agonists to simulate potent immune responses due to the rapid dissolution may be resolved following their covalent attachment to antigens (e.g. conjugation of CpG oligodeoxynucleotide (ODN) onto HIV-1 Gag protein enhances the induction of adaptive immunity).[27,28]

This review briefly summarizes different TLR agonists used as immunostimulatory adjuvants, along with their mechanisms of action, and provides an overview of different approaches used to develop antigen-TLR ligand conjugates. For each approach, different examples of TLR agonists that have been used in preclinical or clinical trials are provided. This review focuses on semisynthetic strategies, which include chemical ligation and enzyme-mediated ligation approaches.

TOLL-LIKE RECEPTOR LIGANDS AS IMMUNOSTIMULATORY ADJUVANTS

Adjuvants are designed to improve the immunogenicity of antigens in vaccines, by stimulating the immune system (i.e. immunopotentiators) or improving their delivery to antigen presenting cells (i.e. delivery systems).[8] They are generally required to elicit protective immune responses against subunit and inactivated whole organism vaccines. Ideal adjuvants should possess properties such as the ability to prolong the duration of immune responses, as well as: stimulating rapid protective immune responses following an infection; stimulating both humoral and cell mediated immune responses; and reducing the cost of vaccination by reducing the required antigen dosage.[8] The mechanism of action of adjuvants can be divided into two categories: 1) particulate vaccine delivery systems that deliver antigens to antigen presenting cells through which both humoral and cell mediated responses are enhanced, and 2) immunostimulatory adjuvants, which stimulate cells directly via specific receptors leading to quicker, more specific, and more potent immune responses.[1,8]

Toll-Like Receptors. The innate immune system plays a fundamental role in protecting against bacterial, viral and parasite infections through the recognition of pathogen-associated molecular patterns (PAMPs) by PRRs.[29] PAMPs are molecules produced by pathogens, that are not produced in the host, which activate the innate immune system to identify non-self molecules. There are several different types of PRRs that are associated with the early detection of pathogens following infection including TLRs, C-type lectin receptors (CLRs), RNA-sensing RIG-I (retinoic acid-inducible gene I)-like receptors (RLRs) and NOD-like receptors (NLRs). TLRs are transmembrane proteins, usually expressed by DCs, macrophages and B cells, which play a critical role in both the innate and adaptive immune responses. To date 13 TLRs have been classified, of which TLR1-10 have been identified in humans, and TLR1-9 and 11-13 have been identified in rodents. TLRs and interleukin-1 receptors (IL-1Rs) belong to the same superfamily, TLR/interleukin-1 receptors (TIRs), and share the same intracellular homology domain.[30] The extracellular domains of TLRs are involved in the recognition of PAMPs.

TLR1, 2, 5 and 6 are located on the surface of cells, with signaling through these receptors inducing T_H2-type immune responses. In comparison, TLR3, 7, 8 and 9 are located on the

membrane of endosomal compartment and signaling via them induces T_H1-type immune responses. TLR4 is expressed on both the cell surface and endosomal membranes. Surface expressed TLR4 signaling induces the production of proinflammatory cytokines, but can be switched to endosomal signaling under regulation of the p110δ isoform of phosphatidylinositol-3-OH kinase (PI(3)K), which results in the generation of anti-inflammatory cytokines.[31] Finally, TLR10 is expressed on the cell surface but its ligand/s and functions are currently unknown.[32] Based on their location, surface expressed TLRs are responsible for the detection of extracellular bacterial cell wall components[33-36] while endosomal TLRs are associated with the recognition of intracellular nucleic acids from viruses and bacteria.[37-41]

Toll-Like Receptor Signaling Pathways and Adjuvant Mechanisms. Following the binding of their ligands, TLRs undergo a conformational change by forming homo- and heterodimers which are necessary to trigger intracellular signaling. During this process, different adaptor proteins are recruited to the intracellular TIR domain, which are essential to drive the subsequent signaling. Four adaptor proteins have been identified including: myeloid differentiation primary response factor 88 (MyD88), TIR domain-containing adaptor protein (TIRAP, also known as Mal or MyD88 adaptor like protein), TIR domain-containing adapter-inducing interferon-β (TRIF, also known as TICAM-1) and TRIF-related adaptor molecule (TRAM, also known as TICAM-2).[42] As shown in Figure 1, there are two signaling pathways responsible for the activation of intracellular cascades. These are the MyD88-dependent and MyD88-independent (also known as the TRIF-dependent) pathways.[43] All TLRs except TLR3 require MyD88 as the essential adaptor protein. TLR4 signaling can activate both MyD88-dependent and MyD88-independent pathways in association with the coreceptors CD14 and MD2, which are required for the recognition of its ligand lipopolysaccharide (LPS). In addition, the biological functions of TLR10 are not fully understood, although TLR10 has been reported to show inhibitory properties that control inflammatory responses mainly induced by TLR2.[32]

The expression of TLRs by DCs, macrophages and B cells provides a means to uptake antigen when presented in the context of TLR ligands for presentation via major histocompatibility complex (MHC) I and II, thereby enhancing the production of antigen-specific antibodies and CTL responses. TLR signaling ultimately leads to the induction of pro- and anti-inflammatory mediators, which results in the development and proliferation of T cells, as well as promotion of memory T cells. The above features of TLRs make their ligands perfect candidates as immunostimulatory adjuvants in the context of vaccination.

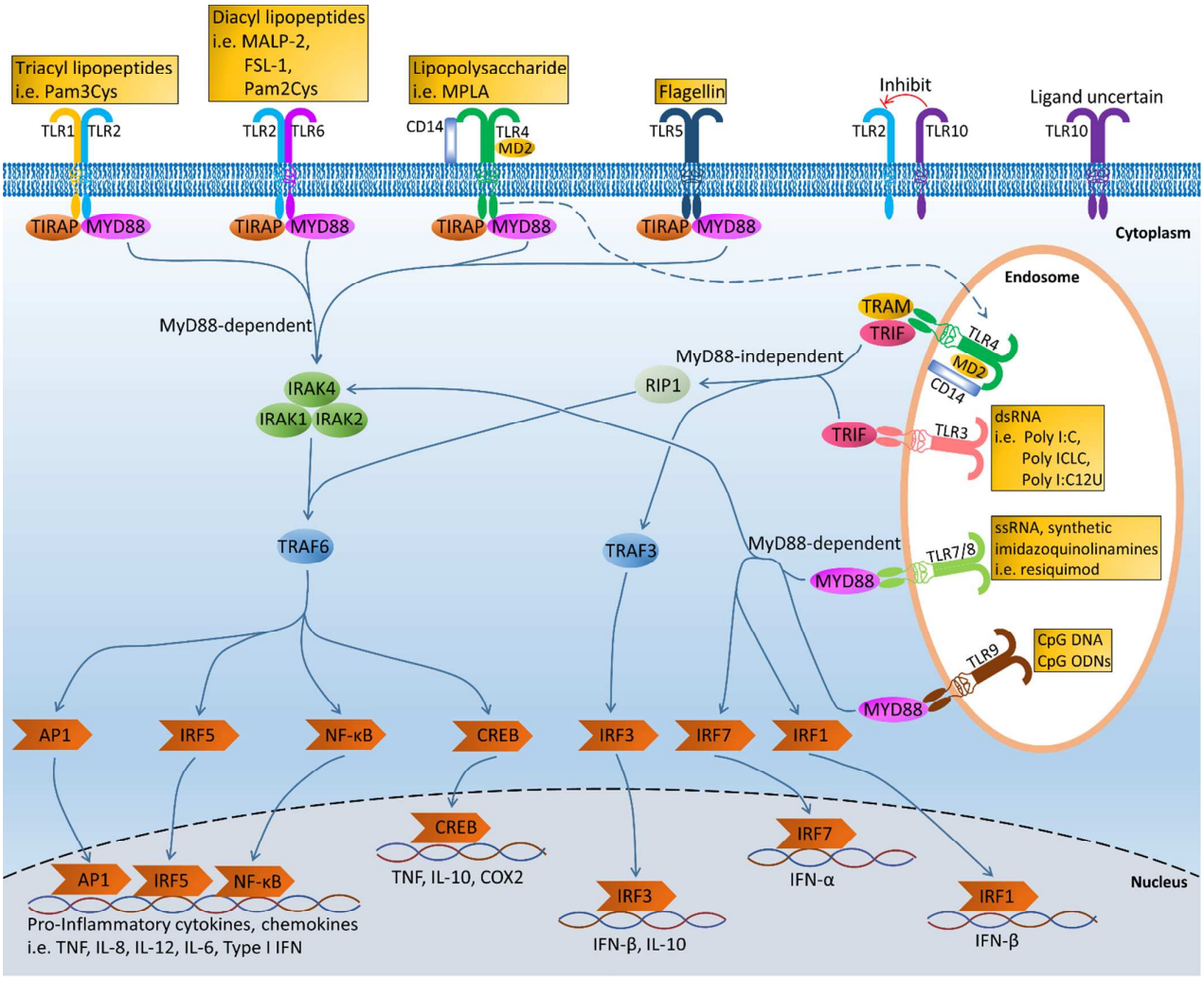


Figure 1. TLR signaling pathways of common TLR ligands. TLRs can detect exogenous PAMPs, resulting in the activation of TLR signaling pathways that lead to the regulation of transcription factors and the release of proinflammatory cytokines and chemokines. In the MyD88-dependent pathway, MyD88 is recruited to the cytoplasmic TIR domain to activate interleukin-1 receptor-associated kinase (IRAK) 4 and IRAK1/2, followed by activation of tumor necrosis factor receptor-associated factor 6 (TRAF6).[43] Ultimately, this pathway leads to nuclear translocation of transcription factors including activator protein 1 (AP1) and nuclear factor-κB (NF-κB) which mediate induction of pro-inflammatory cytokines and chemokines such as TNF, IL-6, 8, 12 and type I interferon (IFN). The MyD88-dependent pathway also results in the translocation of cAMP response element-binding protein (CREB) which mediates induction of TNF, IL-10 and cyclooxygenase-2 (COX-2). For MyD88-dependent signaling TLR1, 2, 4 and 6 require the cooperation of TIRAP while TLR 7, 8 and 9 do not.[42] TLR3 recruits TRIF to activate TRAF3 and thereafter interferon regulatory factor (IRF) 3 which results in the induction of IFN-β and IL-10. Unlike TLR3, TLR4 needs to firstly recruit TRAM before activating TRIF when signaling through the TRIF-dependent pathway. TLR7, TLR8 and TLR9 signaling leads to nuclear translocation of AP1 and NF-κB in MyD88-dependent pathway and activation of IRF7 and IRF1 that ultimately results in secretion of cytokines and chemokines such as TNF, IL-6, 12 and IFN-α, IFN-β. Overall TLR signaling results in host cell responses such as the induction of inflammatory mediators and production of antimicrobial agents. Many TLR agonists have been identified. For example, triacylated lipopeptide tripalmitoyl-*S*-glyceryl cysteine

(Pam3Cys) as a TLR1/TLR2 heterodimer agonist;[24,26] diacylated lipopeptide dipalmitoyl-*S*-glyceryl cysteine (Pam2Cys), *M. fermentans* macrophage-activating lipopeptide (MALP-2) and fibroblast-stimulating lipopeptide (FSL)-1 as TLR2/TLR6 heterodimer agonists;[24] dsRNA and Poly I:C as TLR3 agonists;[23] MPLA as a TLR4 agonist;[25] bacterial heat shock protein complexes (HSPCs) including HSP70, HSP90 and gp96 possibly targeting TLR2 and TLR4;[44-46] Bacterial flagellin as a TLR5 agonist;[18] Imiquimod as a TLR7 agonist; ssRNA, gardiquimod, and resiquimod as TLR7/TLR8 heterodimer agonists; and CpG DNA and CpG ODNs as TLR9 agonists.[21,22]

Examples of Toll-Like Receptor Agonists as Immune Adjuvants. Several vaccines have been recently marketed, which feature TLR agonists as immunostimulatory adjuvants. An important recent example is Trumenba®, which includes two *Neisseria meningitides* factor H protein binding proteins that incorporate a covalently-linked, N-terminal bacterial lipopeptide TLR2 adjuvant similar to Pam3Cys.[47] In comparison, the most recent adjuvant mixture approved by the US Food and Drug Administration (FDA) is AS04, an immunostimulatory adjuvant licensed in 2005 by GlaxoSmithKline (GSK).[48] The major component of AS04 is MPLA, a derivative of the TLR4 agonist LPS. LPS consists of a hydrophobic lipid A component and a hydrophilic polysaccharide.[48] TLR4, together with co-receptors CD14 and MD2, recognizes LPS through its lipid A component, activating inflammatory pathways.[49] This can lead to septic shock, and as such LPS cannot be used directly as an adjuvant in humans due to its high toxicity.[31] Consequently, derivatives that do not possess this toxicity have been developed. MPLA is a detoxified analog which has a thousand times lower toxicity compared to LPS. AS04 consists of MPLA and aluminum hydroxide (AH) or aluminum phosphate (AP) depending on the vaccine design, and has been widely evaluated in many vaccines against viral diseases (e.g. hepatitis B virus (HBV), herpes simplex virus (HSV), human papillomavirus (HPV) and Epstein-Barr virus (EBV)). Fendrix (HBV) and Cervarix (HPV) are successful examples of commercial AS04-adjuvanted vaccines.[48] Fendrix, developed by GSK and licensed by the FDA in 2005, has demonstrated its ability to induce longer lasting and higher titer antigen-specific antibodies compared to aluminum-adjuvanted vaccines, and elicits enhanced CTL responses in humans. Cervarix, containing HPV types 16 and 18 antigens, has been approved for use in Australia and the USA in 2007 and 2009, respectively. This vaccine has been shown to induce a long-lasting immune response against both HPV-16 and -18 in females aged 9-25 years.[48] Administration of both vaccines revealed that the high-levels of antibodies induced by AS04 also show strong virus neutralization capacity. The success of AS04 has paved the way for the utilization of other TLR agonists as marketable adjuvants.

MPLA has also been intensively investigated as an adjuvant on its own. For example, Pollinex Quattro is an MPLA-adjuvanted vaccine that is used for seasonal allergic rhinitis therapy in Europe,[50] and is progressing towards FDA approval. Further, MPLA has also been used in combination with *Quillaja saponaria* 21 (QS21) and liposomes to compose AS01, which is used in the first licensed malaria vaccine Mosquirix (GSK).[51] Some other TLR agonists that are being investigated in vaccine clinical trials include: 1) Ampligen, a TLR3 agonist, which contains Poly I:C_{12}U (a low toxicity Poly I:C analog) tested in phase I and II trials against ovarian, fallopian tube or primary peritoneal cancer, and tested in phase II and III trials targeting HIV;[23] 2) Flagellin, a TLR5 agonist that has been used in several influenza vaccines

including VAX102,[18] VAX125,[17] and VAX2012Q[19] and tested in phase I and II trials; 3) Entolimod (CBLB502), an engineered flagellin analog tested in phase I studies targeting cancer;[52] and 4) CpG7909 as a TLR9 agonist combined with QS21 and MPLA in a liposomal formulation named AS15 and tested in phase II trials against MAGE-A3-positive melanoma.[25]

COVALENTLY ATTACHED TOLL-LIKE RECEPTOR LIGANDS IN ANTIGEN-ADJUVANT CONJUGATE VACCINES

With the development of (bio)conjugation techniques, TLR ligands have been covalently attached to a number of molecules, from synthesized peptides to expressed proteins and even intact viruses.[53] Normally antigens are delivered as a mixture with adjuvants. However, simple mixing cannot guarantee that delivered antigens will be recognized and presented by APCs, as the adjuvant and antigen may dissociate after administration. On the contrary, antigen-TLR ligand conjugates help to ensure that both antigen and adjuvant reach APCs simultaneously; are recognized by surface receptors on APCs; and are internalized together. Thus, more potent immune responses can be achieved by this approach when compared to those induced by antigen-TLR ligand mixtures. This in turn allows for the vaccine dose to be reduced, decreasing the likelihood of adverse effects. Since protein and peptide based antigens are produced by different approaches, various conjugation approaches have been developed to ensure the utility of these approaches for different antigen types. In this section, three covalent attachment strategies (e.g. recombinant fusion proteins; chemical conjugation; enzyme-mediated conjugation) that are most often utilized to produce protein and peptide antigen-TLR ligand conjugates will be discussed.

Role of Toll-Like Receptor Ligands as Covalently Attached Immunostimulatory Adjuvants. TLR ligands, as immunostimulatory adjuvants, have multiple immunomodulatory functions including: modulation of APC migration and activation; up-regulation of costimulatory molecules on APCs for better antigen processing and presentation; induction of T_H1, T_H2 or T_H17 responses through the polarization of $CD4^+$ T cells; priming of $CD8^+$ T cells for antigen-specific CTL responses; development of antibody responses and activation of natural killer (NK) cells (Figure 2).[43,44,47,54-58] However, the co-administration of mixtures of TLR ligands and antigens may not elicit desired immune responses, due to several reasons: 1) dissociation of the TLR ligands can occur rapidly after injection;[27] 2) it is more difficult to deliver free antigens to TLR receptor-expressing APCs for presentation than to deliver antigens linked to TLR ligands; and 3) antigen adjuvant mixtures tend to elicit lower potency immune responses compared to their fusions. In addition, many studies have confirmed that both innate and adaptive immune responses are generated with increased efficiency when antigens are covalently linked to TLR ligands.[27,59] As shown in Figure 2, conjugated antigens are taken up by DCs more efficiently, thereby enhancing antigen presentation. Additionally, prolonged CTL responses are induced after antigen conjugates have been internalized by DCs through receptor-mediated endocytosis.[59] This may be due to the formation of an intracellular antigen depot in DCs (Figure 2), containing the antigen conjugate, which leads to the continuous presentation of antigenic peptides (after antigen conjugates have been slowly released from the depot and processed) to T cells, thereby leading to long-term priming capacity.[59]

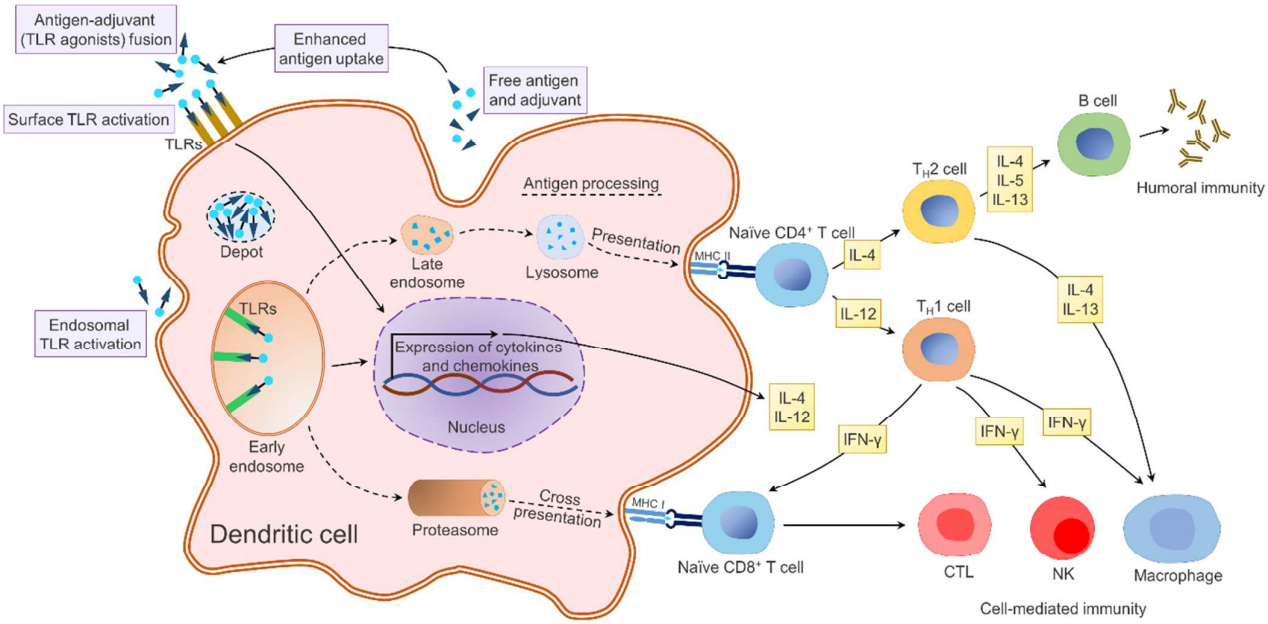


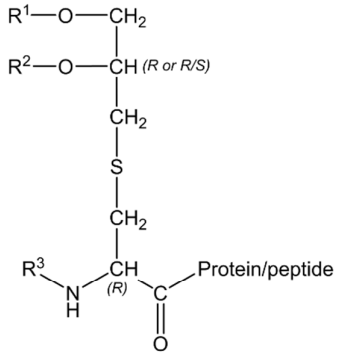
Figure 2. Putative mechanisms for the enhanced immunity associated with covalently attached TLR agonists. Administration of antigen-adjuvant fusions enables both antigen and TLR agonists to be delivered to the same DC at the same time, hence promoting efficient antigen presentation after conjugated antigens have been taken up by DCs. After internalization, TLR component helps to activate transcription factors that induce the production of cytokines and chemokines, such as IL-4 and IL-12, which help direct specific immune responses (e.g. T_H1, T_H2 and CTL type responses). Conjugated antigens are then processed in DCs, and their antigenic peptides are presented in the context of MHC class I or II molecules through presentation or cross presentation by DCs, which leads to the maturation of native $CD4^+$ and $CD8^+$ T cells.[60-62] $CD4^+$ T cells play a major role in shaping the adaptive immune system through: 1) promoting B cell maturation, which leads to the generation of memory B cells and long-lived plasma cells; 2) secreting different types of cytokines (e.g. IL-4, 5, 13 and IFN-γ), which play different roles in the development, maturation, and activation of macrophages and NK cells; and 3) activating $CD8^+$ T cells and regulating CTL responses, which are central for host defenses against intracellular pathogens as well as the elimination of cancer cells.[63-65]

Antigen-Toll-Like Receptor Ligand Conjugates Expressed as Recombinant Fusion Proteins. With the development of recombinant DNA technology, it is convenient to express antigen-TLR ligand conjugates in various hosts e.g. *Escherichia coli* (*E. coli*), yeast, insect and mammalian cells. As a well-established and cost-effective expression system, *E.coli* is the most popular system for the expression of heterologous proteins in a high yield. There are several advantages of using *E.coli* as an expression system: exogenous DNA can be transformed rapidly and easily; growth media are well defined and affordable; cells can grow to high density within a short period of time; and soluble expressed proteins carrying an affinity tag can be easily purified. On the other hand, some proteins may not express, express as insoluble aggregates (inclusion bodies), or express poorly (proteins with high molecular weight e.g. >75kDa) in this system. This may be due to protein toxicity, which interferes with normal *E.coli* growth, as well as codon bias which leads to depletion of low-abundance tRNAs. Also, if the proteins were expressed as inclusion bodies, it can be extremely difficult

to refold them back to their natural conformation. Furthermore, as a Gram-negative bacteria, *E.coli* produces endotoxins, which can elicit strong immune responses, sometimes leading to septic shock. Thus the chromatographic methods followed should be carefully selected to remove endotoxins during protein purification steps.

Many TLR ligands have been investigated as fused recombinant proteins expressed by *E.coli*. The most successful example is Trumenba®, which is a self-adjuvanting (TLR2 agonist) lipoprotein vaccine against Meningococcal Meningitis group B.[47] Bacterial flagellin (a TLR5 ligand) has also been intensively studied in clinical trials as fusions with influenza antigens.[17-19] Other examples tested in preclinical studies include Ag473, a TLR2 ligand fused to dengue virus envelope protein E3;[66] HSP70 fused to HIV-1 p24 protein;[44] and the type III repeat extra domain A from fibronectin (EDA) as TLR4 ligand fused to chicken ovalbumin (OVA) epitope.[45,53,66,67] All the above examples will be discussed in the following paragraphs.

Trumenba® is the first vaccine approved by the FDA to protect against invasive disease caused by *Neisseria meningitidis* serogroup B (NMB) in individuals aged 10 to 25.[47] Trumenba® is composed of two lipidated fHBP variants, A05 from subfamily A and B01 from subfamily B, namely rLP2086-A05 and rLP2086-B01, respectively.[68] fHBP, a key virulence factor also known as LP2086, is a conserved surface-exposed lipoprotein responsible for evasion from complement-mediated killing through binding to human complement factor H.[68,69] rLP2086-A05 and rLP2086-B01 are both recombinantly expressed in *E.coli*, with an N-terminal lipid tail as per the native lipoproteins (Figure 3A).[47] As a TLR2 agonist, the lipid moiety is able to elicit broader immune responses against the antigens when compared to their non-lipidated forms.[47] Clinical trials revealed that Trumenba® was well tolerated and able to stimulate immune responses against a broad variety of group B meningococcal strains prevalent in the USA.[68] Trumenba® represents the first vaccine consisting of a covalently attached TLR ligand and a protein antigen.



A Trumenba:
R^1, R^2=Mainly $CH_3(CH_2)_{14}CO$, or $CH_3(CH_2)_5CH=CH(CH_2)_7CO$, or $CH_3(CH_2)_5CH=CH(CH_2)_9CO$
R^3=$CH_3(CH_2)_{14}CO$

B Ag473:
R^1, R^2=Mainly $CH_3(CH_2)_{14}CO$, or $CH_3(CH_2)_5CH=CH(CH_2)_8CO$, or $CH_3(CH_2)_{16}CO$, or $CH_3(CH_2)_6CH=CH(CH_2)_8CO$
R^3=H or $CH_3(CH_2)_{14}CO$

C Pam2Cys:
R^1, R^2=$CH_3(CH_2)_{14}CO$, R^3=H

D Pam3Cys:
R^1, R^2, R^3=$CH_3(CH_2)_{14}CO$

Figure 3. Schematic structure of bacterial lipo-peptides/proteins (TLR1/TLR2 or TLR2/TLR6 agonists). (A)

Trumenba®, (B) Ag473, (C) Pam2Cys and (D) Pam3Cys.

Antigen-adjuvant fusion vaccines incorporating *Salmonella typhimurium* flagellin type 2 (STF2) have also been tested in multiple clinical studies. Flagellin, a natural ligand of TLR5, is the major component of flagella in bacteria, and features four globular domains (0, 1, 2, and 3) where domain 1 is recognized by TLR5 (Figure 4A).[70] Flagellin fusion vaccine development strategies have included: 1) antigen of interest attached to the N- or C-terminus; 2) antigen of interest replaces the D3 domain; 3) antigen of interest inserts into the D3 domain; and 4) a combination of any of the above.[17-19] A phase I study using VAX102, a recombinant fusion protein consisting of four repeats of matrix protein 2 (M2e) linked to flagellin (Figure 4B), has been completed and the results showed that VAX102 was well tolerated at doses up to 1 µg, with a four-fold increase in serum IgG anti-M2e antibody titers observed when compared to subjects who received placebo.[18] In addition, a phase II trial targeting adults aged 65 years and older has been completed for VAX125, a recombinant fusion protein composed of flagellin and a hemagglutinin globular head of the HA1 domain from strain A/Solomon Islands/3/2006 (Figure 4C). The results revealed that VAX125 is safe for in this group at doses up to 8 µg. Furthermore, a 10-fold increase in serum hemagglutination-inhibition (HAI) antibody geometric mean titer (GMT) was generated by VAX125 which is much better than 2-fold increase, observed with the standard Fluzone vaccination.[17] Finally, a phase II study of VAX2012Q, a quadrivalent influenza vaccine containing four different types of hemagglutinin globular head subunits (strain A/California/07/2009, A/Perth/16/2009, B/Wisconsin/01/2010, and B/Bangladesh/5945/2009) each of which is inserted within the D3 domain, or in place of the D3 domain, or in place of D3 and at the C-terminus, has recently been completed in adults aged 18-64 years. The results of this study demonstrated no serious safety issues at all dose levels and seroprotective immune responses were elicited.[19]

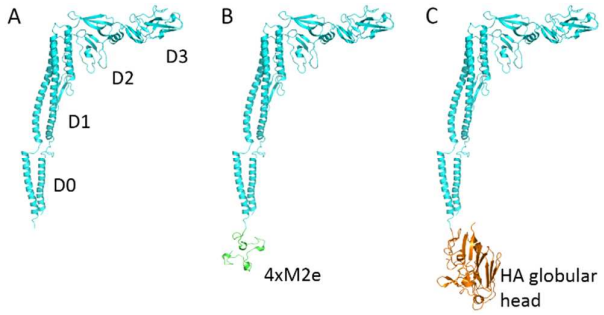


Figure 4. Schematic representation of flagellin and flagellin fusion vaccine candidates. (A) Flagellin. (B) VAX102, flagellin (cyan) with the C-terminus fused with four tandem copies of the ectodomain of the M2e (green). (C) VAX125, flagellin (cyan) with the C-terminus fused with a globular head of the HA1 domain (orange).

Lipidated meningococcal antigen Ag473 is a bacterial lipoprotein encoded by the NMB1468 gene.[71] The structure of recombinantly expressed Ag473 in *E. coli* has been characterized, as shown in Figure 3B.[72] As a TLR2 agonist, Ag473 has the ability to activate DCs and promote their maturation. Furthermore, genetic fusions of the N-terminus of Ag473 to other antigens provide a method to recombinantly produce vaccines incorporating an N-terminal bacterial lipoprotein adjuvant. Immunization of mice with rlipo-D1E3 (envelope protein domain 3 from

dengue virus fused with the N-terminal 40-amino acids of Ag473) expressed in C43 (DE3) *E. coli* (which is necessary to produce the lipidated N-terminus) elicited higher titers of anti-E3 IgG antibodies than observed with E3 alone or alum adjuvanted E3.[66] The results also showed that rlipo-D1E3 elicited significantly more neutralizing antibodies against dengue virus-2 compared to E3 adsorbed on aluminum phosphate.[66]

The ability of HSPs to act as danger signals and induce T_H1 and T_H17-type responses makes them potential adjuvants for fusing with protein or peptide antigens.[73,74] r4M2e.HSP70c was constructed and expressed in *E.coli* by linking four tandem repeats of influenza A virus M2e with a *Mycobacterium tuberculosis* C-terminal truncated HSP70.[75] Compared with r4M2e, rHSP70c, or r4M2e adjuvanted with alum, r4M2e.HSP70c induced stronger and longer lasting humoral and cellular immune responses characterized by the enhanced production of M2e-specific IgG2a antibodies and the T_H1 cytokine IFN-γ.[75] Another study using murine HSP70 fused with HIV-1 p24 protein was conducted in BALB/c mice. The results demonstrated that HSP70 enhanced p24 protein engulfment by DCs and stimulated the activation of DCs. P24-specific IgG2b antibodies and IFN-γ were induced at a much higher levels compared to those induced by p24 alone.[45]

Fibronectins are a group of extracellular matrix glycoproteins which participate in many pathological and physiological processes, especially those associated with fundamental cell processes such as cell growth, cell adhesion, cell migration and wound healing.[76,77] EDA, encoded by an alternatively spliced type III exon, has been identified to exhibit the ability to stimulate TLR4 activity,[78] which suggest that EDA could potentially be used as a vaccine adjuvant.[67] Intravenous administration of an EDA fusion with an OVA $CD8^+$ T cell epitope peptide to C57BL/6 mice demonstrated that OVA-specific CTL responses were induced by the fusion protein, but not by OVA $CD8^+$ T cell epitope alone. In addition, proinflammatory cytokines (e.g. IL-12 and TNF-α) were secreted by by bone marrow (BM)-derived DCs following stimulation with the recombinant EDA proteins. These immune responses were sufficient to protect mice from challenge by OVA tumor cells. Further, EDA has been demonstrated to adjuvant high molecular weight proteins through the attachment of full length OVA to the C-terminus of EDA. This construct demonstrated similar enhancement of immunogenicity towards the attached OVA protein compared to the peptide antigen, demonstrating that EDA has the potential to be utilized as an adjuvant for the development of antigen-adjuvant fusion protein vaccines.[67]

Semisynthetic Approaches to Attach Immunostimulants onto Antigens. Some TLR ligands cannot be expressed as recombinant proteins, hence they are chemically synthesized and attached to peptide or protein antigens. There are two commonly used covalent attachment approaches, chemical ligation and enzyme-mediated ligation. For classical chemical ligation approaches to be used successfully, the reactive site (e.g. a cysteine residue) must be readily accessible (i.e. not buried within the proteins 3D structure) to enable the conjugation reaction to proceed efficiently. Therefore, on occasion ligation reactions must be performed under denaturing conditions (e.g. using chaotropes such as 6 M guanidine or 8 M urea) in order to expose the reaction site. This limits their use for many protein antigens, for which correct folding is often a requirement to ensure the generation of appropriate immune responses. In addition, the rate and extent of the reaction between each component can be

greatly affected by the concentration of each component that can be achieved under the reaction conditions. Thus, denaturing conditions may be used to help prevent the aggregation and precipitation of each reactant at high concentrations, in order to allow for high yielding conjugation reactions. Further, in some cases, the use of lower concentrations of chaotropes (e.g. 2 M urea) may allow for a balance to be achieved between maintaining the solubility of each component and minimizing protein unfolding. While these conditions can benefit the rate and extent of chemical ligation approaches, enzyme-mediated ligations generally need to be conducted under native conditions in order to maintain the activity of the enzymes used to achieve the conjugation reaction.

Chemical Ligation. Conventional chemical ligation techniques involve the use of thiol- or amine-reactive reagents, maleimides or *N*-hydroxysuccinimidyl (NHS) esters respectively, to form conjugates through the side-chain of cysteine or lysine residues (Figure 5A and 5C). Tyrosine, aspartic acid, glutamic acid residues and the N- or C-terminus of polypeptides have also been utilized as modification sites.[79] Although the classic ligation methods are still in widespread use, a variety of approaches that enable site-specific attachment reactions onto proteins have been developed e.g. expressed protein ligation (EPL) which combines native chemical ligation (NCL)[80] and intein to conjugate a synthetic peptide onto a expressed protein (Figure 5B).[81,82] The NCL reaction generates a native peptide bond at the ligation site through a reaction that involves the attack of a thioester containing component by another component containing an N-terminal cysteine residue.[80] This leaves a cysteine residue at the ligation site, which can be converted to an alanine by a desulfurization reaction to prevent the formation of disulfide bonds (Figure 5B).[24,80]

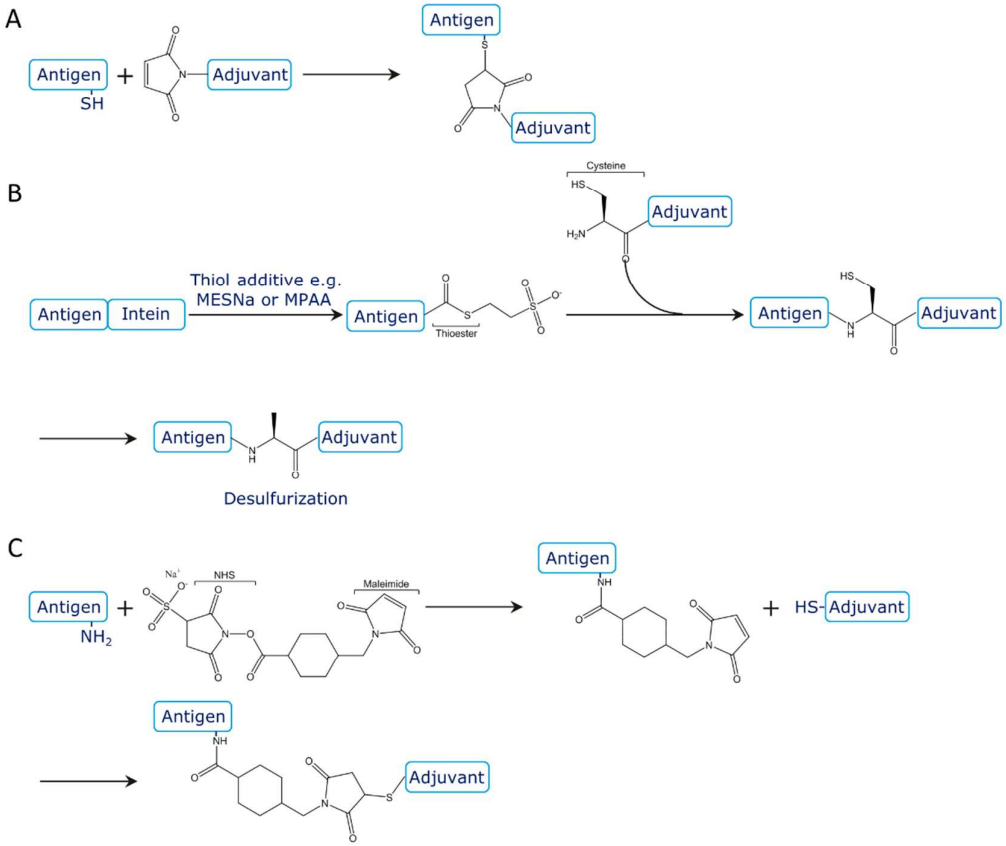


Figure 5. Examples of chemical ligation reactions suitable for the generation of antigen-adjuvant fusions. (A) Maleimide (thioether) ligation. (B) Expressed protein ligation. (C) Sulfo-SMCC (contains both NHS ester and maleimide group) was used in ligation reactions to conjugate the molecule of interest (contains a thiol group) onto antigen protein through lysine side-chains.

The first application using a semisynthetic-ligation approach to conjugate a TLR agonist to an antigen involved the conjugation of OVA and a CpG ODN.[83-85] The synthetic ODN (containing a 5' thiol group) was covalently attached to the OVA protein by modifying multiple OVA amino groups with sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) to form maleimides, followed by reaction with the CpG ODN (Figure 5C). In accordance with the immunological properties of TLR9 agonists, a T_H1-biased immune response was induced in mice following immunization with this vaccine. Further, high-level antigen-specific CTL responses, which conferred protection against a lethal OVA tumor challenge, were demonstrated. This synthetic ODN was further conjugated to two other antigens including *E. coli* β-galactosidase and HIV-1 envelope glycoprotein gp120, the results revealed that T_H1 cells were induced more rapidly, along with stronger CTL responses and greater titers of IgG2a antibodies compared to where antigen-ODN mixtures were administrated in BALB/c mice.[13] Similar techniques have also been used to conjugate OVA to TLR7/8 agonists (i.e. 3M012 and 3M044; 3M pharmaceuticals).[15,16] These OVA-TLR7/8 agonist conjugate vaccines were able to induce the migration of DCs to the lymph node, enhance presentation and cross-priming efficiency, and induce the secretion of type I IFN. 3M-012 has also been conjugated to HIV-1 gag protein and subcutaneous administration of this conjugate in C57BL/6 mice revealed that enhanced gag-specific $CD8^+$ T cell responses were induced, along with increased numbers of cells expressing CD127, a marker correlated with the differentiation of effector cells to memory cells.[28] Isothiocyanate and maleimide derivatives of another TLR7 imidazoquinoline agonist have been covalently attached to model protein antigens such as α-lactalbumin and human serum albumin to evaluate the immunostimulatory profiles of this TLR7-agonistic imidazoquinoline.[86] Intramuscular administration of the α-lactalbumin conjugate and antigen adjuvant mixture in outbred CF-1 mice demonstrated that higher titer antigen specific IgM, IgG1, and IgG2a antibodies were elicited by the conjugate when compared to the mixture and more importantly, IgG antibodies induced by the conjugate were observed to have a higher affinity towards α-lactalbumin.[86]

The TLR3 ligand Poly I:C has been used in a *Mycobacterium tuberculosis* antigen-adjuvant fusion vaccine consisting of Ag85B-HspX (AH) fusion protein and Arabinogalactan (AG).[12] Ag85B, the major protein secreted by actively replicating *M. tuberculosis*, plays an essential role in the virulence of M. *tuberculosis*;[87] is highly immunogenic, and induces a T_H1-type immune response.[88] HspX, a latency-associated antigen and molecular chaperone, is the dominant protein produced by *M. tuberculosis* in latently infected TB patients, and has been reported to be critical in the growth of *M. tuberculosis* following infection.[89,90] AG, approved by the FDA as a food supplement, is a natural polysaccharide that can effectively activate immune responses through prompting splenocytes proliferation, upregulating the expression of various cytokines, and stimulating anti-tumour immunity.[91] Poly I:C was activated by incubation with ethylenediamine and sodium bisulfate. Periodate oxidized AG was incubated

with activated Poly I:C and sodium cyanoborohydride to generate a potent adjuvant AG-P. Recombinant protein AH was then conjugated to AG-P in the presence of excess 1-(3-(dimethylamino)propyl)-3-ethylcarbodimide through the C-terminal carboxyl groups. Intraperitoneal immunization with this compound in C57BL/6 mice revealed that AH-specific IgG antibodies and mixed T_H1/T_H2-type cytokines were elicited at significantly greater levels by AH-AG-P than induced by AH, AH-AG, AH-P, or a mixture of AH/AG-P. The higher efficiency observed with this antigen-adjuvant delivery system was due to two reasons. Firstly, AH is protected from proteolytic digestion through conjugation with AG-P, thereby prolonging the exposure time *in vivo*. Secondly, this approach ensures the simultaneous uptake of both AH and AG-P by DC, thereby increasing the presentation efficiency significantly.

Bacterial lipopeptides and their synthetic agonists have also been shown to be ideal candidates for antigen-TLR ligand conjugate vaccines. TLR2/6 and TLR1/2 heterodimer ligands Pam2Cys (Figure 3C) and Pam3Cys (Figure 3D) are typical examples which have been chemically attached to antigens.[24,92] For example, we observed that administration of a Pam2Cys, Pam3Cys or bacterial lipopeptide analog (lipid core peptide (LCP)) that was conjugated using EPL or maleimide chemistry onto a multiantigenic model group A *streptococcus* (GAS) antigen, containing a broadly protective (J14) and 7 strain-specific (N-terminal M protein peptides) peptide antigens, significantly enhanced production of antigen specific IgG antibodies.[24] The Mxe GyrA Intein, fused to the same GAS antigen on the C-terminus, can be converted to a reactive α-thioester in several steps. Following that, lipid adjuvant peptides were conjugated to the recombinant thioester antigen through NCL in the presence of 6M guanidine. In this case utilization of denaturant will not cause issues since the GAS polytope antigen is mainly composed by linear peptide antigens without structure. Results showed that persistent antigen specific IgG antibodies were rapidly elicited after immunization. The major issue when performing conjugation in this case is the high hydrophobicity of the lipopeptides, which led to insoluble precipitates in aqueous solvents during preparation. It is very common to utilize denaturant such as organic solvent and chaotropic agent to improve the solubility of lipid adjuvants, which unfortunately will most probably result in disruption of native structure of the administrated antigens.

Enzyme-Mediated Ligation. Enzyme-mediated ligation reactions have attracted significant attention in recent years due to their many advantages, which include the ability to simply and efficiently, site-specifically modify proteins, under conditions that maintain their native structure. This is highly significant, as the majority of the antigens reported in the literature are folded protein antigens. A number of enzymes have been successfully used for bioconjugation reactions with proteins. These include: *Staphylococcus aureus* sortase A (SrtAsa)[93] and *Streptococcus pyogenes* sortase A (SpSrtA);[94,95] *Clitoria ternatea* butelase 1;[96,97] *Oldenlandia affinis* (*O. affinis*) asparaginyl endopeptidase (OaAEP);[98-100] SpyLigase;[101,102] transglutaminase (TGase);[103,104] phosphopantetheinyl transferases (PPTases);[105] biotin ligase;[106] and lipoic acid ligase.[107] In the following paragraphs, a brief description of some of the leading enzyme-mediated ligation approaches (OaAEP, butelase 1 and sortase A) will be provided.

The asparaginyl endopeptidase, $OaAEP1_b$,[98] represents one of the most recently identified

enzymes used for enzyme-mediated ligations. It was identified from *O. affinis*, a plant that produces cyclotides (small cyclic peptides, characterized by a cyclic cystine knot motif). During cyclotide maturation, the N- and C-terminal propeptides of the linear cyclotide precursor are cleaved, followed by ligation of the N- and C-terminus by a cyclase. $AEP1_b$ is a cyclase responsible for cyclotide generation in *O. affinis*. It is readily overexpressed in *E. coli*, and also possesses the capacity to cyclize unrelated peptides/proteins (Figure 6A),[98] thus allowing for its use as a ligase enzyme for protein and peptide ligation reactions.[100] The ligation reaction also benefits from the requirement for the presence of relatively short flanking sequences on each peptide/protein component, with highly efficient ligations observed where one species possesses a C-terminal Asn-Gly-Leu motif, and the other two N-terminal flanking residues Gly/Lys/Gln-Leu (Figure 6B).[98,100] However, despite these favorable characteristics, the purification of $AEP1_b$ is complicated; requires proteolytic activation at pH 4.5; and is low yielding (~ 1.8 mg/L). Further, ligation reactions are slow, taking up to 22 h to complete, which may impede its widespread application. A mutant OaAEP1 (Cys247Ala) has been identified recently, with significantly increased ligase activity around 160 times higher compared to WT OaAEP1, which will greatly extend its applications.[108]

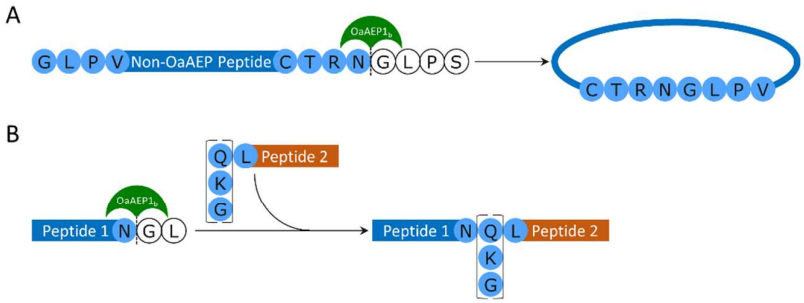


Figure 6. Schematic representation of $OaAEP1_b$-mediated ligation. (A) Non-OaAEP peptides can be cyclized when fused to the recognition motifs present in the *O. affinis* cyclotide precursor. Peptide contains the C-terminal recognition sequence Cys-Thr-Arg-Asn-Gly-Leu-Pro-Ser and the N-terminal recognition sequence Gly-Leu-Pro-Val in this example. (B) For efficient ligation, C-terminal and N-terminal recognition sequences can be simplified to Asn-Gly-Leu and Gln/Lys/Gly-Leu, respectively.

Another recent addition to the enzyme-mediated ligation toolkit is Butelase 1 from the tropical plant *Clitoria ternatea*.[97] This enzyme, like AEP1b, is a cyclase. Further, it has been reported to be the fastest-acting ligase known to date, and has been used in various studies for the ligation or cyclization of peptides and proteins.[96,109,110] The required flanking sequences are also simple, e.g. an Asp/Asn-His-Val motif at the C-terminus of peptide 1 and a Xaa-Ile/Leu/Val/Cys motif at the N-terminus of peptide 2 (Figure 7; where Xaa is any amino acid except proline). The catalytic efficiency for typical butelase-mediated reactions is very high, with only 5 min needed for peptide cyclisation compared to 22 hours with WT $OaAEP1_b$. Thus, these advantages make butelase 1 an ideal enzyme for peptide and protein engineering. However, butelase 1 is currently unable to be expressed in *E. coli*, and thus needs to be extracted from *Clitoria ternatea*. As there is no built in affinity purification handle, a complex 3-day, 4-step chromatography procedure is required to obtain pure butelase 1.[96]

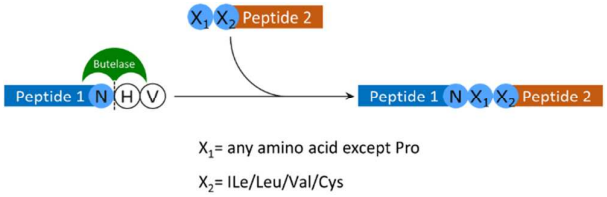


Figure 7. Schematic representation of Butelase-mediated ligation.

Finally, sortase A has been extensively evaluated as a ligase and cyclase for biotechnology applications.[93,94,111] Sortases are transpeptidase enzymes that are found in the cell envelope of almost all Gram-positive bacteria, where they are responsible for the covalent attachment of proteins to the cell wall peptidoglycan layer.[111] Sortase A, also known as the housekeeping sortase, recognizes a Leu-Pro-Xaa-Thr-Gly sequence, which it cleaves between the Thr and Gly residues to form a thioester intermediate (Figure 8; where Xaa is any amino acid). The thioester is then attacked by an N-terminal glycine amine to generate a native peptide bond between the Thr carboxyl group and the Gly amino group.[111] Kinetic analysis of sortase transpeptidation reactions revealed that N-terminal oligoglycine is required for efficient SrtAsa-mediated ligations.[112,113]

As the first sortase to be described, SrtAsa is also the most thoroughly characterised and commonly used for ligation and cyclization reactions. Further, it can be efficiently expressed and purified in *E. coli*. However, the catalytic efficiency of SrtAsa is poor, possibly due to the $K_{m\ LPXTG}$ is relatively high, about 8.7mM.[114,115] Thus, a directed evolution strategy has been used to improve its catalytic efficiency.[116] After eight rounds of screening, a SrtAsa variant containing five mutations (P94R/D160N/D165A/K190E/K196T) was isolated, which demonstrated a 140-fold increase in catalytic activity. We have assessed this enzyme for the site-specific attachment of a synthetic bacterial lipopeptide agonist (TLR2/6 agonist), modified to incorporate an N-terminal tri-glycine, onto a recombinant protein antigen, incorporating a C-terminal LPETG sequence (unpublished data, Z Xu and P Moyle). Use of this enzyme provided access to ligation reactions that proceeded to approximately 90 % completion, over the course of a couple of hours, with approximately 100-fold less sortase enzyme required when compared to the wild-type sequence. This reaction represents the first time that a bacterial lipopeptide TLR2 ligand has been fused to a protein antigen under native conditions, and thus this technique has significant potential to be used for the development of vaccines against a variety of different pathogens.

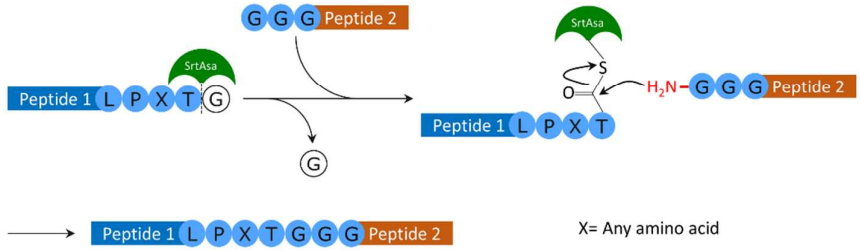


Figure 8. Schematic representation of SrtAsa-mediated ligation.

Synthetic Approaches. As the standard method for synthesizing peptides and small proteins, solid-phase peptide synthesis (SPPS) has been widely used when the desired product is

difficult to express or where unnatural amino acids or other unnatural modifications are desired. Selected B cell, $CD4^+$ or $CD8^+$ T cell epitopes can be economically produced by SPPS in a chemically-defined manner. These synthetic short peptides have the capacity to generate protective immunity following immunization. Vaccines that incorporate one or more synthetic peptide epitopes represent a highly-defined approach to immunization. The covalent attachment of TLR ligands onto these synthetic peptides represents a promising approach to stimulate the innate immune system to induce potent T- and B-cell responses. The selected TLR ligands should be well-defined and their synthesis, or chemical modification should be ammeniable to simple, large scale production. Some lipopeptide TLR2 agonists, such as Pam2Cys and Pam3Cys, contain an amino acid scaffold, and hence can be easily conjugated onto peptide antigens during SPPS.[14,117-120] In addition, TLR7, 8 and 9 agonists have also been conjugated onto peptides to produce self-adjuvanting vaccines.[118,121-123]

Fully synthetic vaccines composed of a model OVA CTL antigen (DEVSGLEQLESIINFEKL; $OVA_{247-264}$) fused with the TLR2 ligand $Pam3CysSK_4$ and TLR9 ligand CpG ODN (5'-TCCATGACGTTCCTGACGTT-3') have been designed and produced with high quality, and without contamination by other TLR ligands such as LPS.[14,118] Uptake and trafficking of these conjugates by DCs demonstrated that although distinct receptors are involved in the uptake of different TLR ligand conjugates, each conjugate is taken up by DCs much more efficiently than unconjugated mixtures both *in vitro* and *in vivo*, indicating that the covalent conjugation is responsible for the enhanced uptake.[118] $OVA_{247-264}$ was also fused to $Pam3CysSK_4$ derivatives by the same group. After immunizing naïve C57BL/6 mice, the OVA epitope specific $CD8^+$ T cell responses were significantly higher in mice immunized with the TLR ligand-peptide conjugates compared to mixtures of the peptide and TLR ligand, indicating that conjugates were efficiently introduced into the MHC I cross-presentation pathway.[14]

Normally TLR7/8 ligands are conjugated onto proteins or peptides through N-terminal or lysine side chain amino groups.[121,122] In such cases, the homogeneity of final product may cause concern when multiple reaction sites are present in antigens. A novel TLR7/8 agonist, norleucine containing an imidazoquinoline moiety on its side chain, has been developed and this compound can be attached to different peptide antigens in a molecular-defined manner during SPPS.[123] This molecule was covalently attached to influenza A virus M2e peptide and the conjugates were subcutaneously injected in mice. Results revealed that M2e-specific antibody responses were induced by the peptide conjugates, but not by M2e or equimolar M2e and TLR7/8 ligand mixtures.[123]

Numerous studies have investigated the conjugation of TLR2 agonists onto different virus antigen epitopes.[119] For example, a HSV glycoprotein B (gB) CTL epitope fused with the Pan DR epitope (PADRE, a universal $CD4^+$ helper T cell epitope) has been synthesized and linked to three palmitic acid moieties to comprise a T_H-CTL lipopeptide.[119] Results from intravaginal administration of this lipopeptide in mice have shown that HSV-specific effector and memory CTL responses as well as T_H1 biased cytokines were induced. Furthermore, immunization with lipopeptide conjugates instead of peptides alone conferred protection in mice against genital HSV-2 challenge. TLR2 ligand-synthetic peptide conjugates have also been tested in clinical studies.[120,124] Two HPV16 synthetic long peptides (SLPs), harboring multiple $CD4^+$ and $CD8^+$ T cell epitopes that cover two most immunogenic regions of E6 oncogenic protein

respectively, have been covalently attached to Amplivant (AV) under GMP environment.[120] Amplivant is a TLR2 agonist Pam3CSK4 based ligand with modification on one of the lipid tails to enhance its binding capacity to TLR2. Both AV-SLP conjugates have the capacity to stimulate the maturation of human DCs *in vitro*. Enhanced secretion of chemokines such as IL-8, IL-10, MCP-1, CCL3, and CCL4 was observed *in vivo* when administrated with AV-SLPs in comparison to SLP alone. HPV16-specific $CD8^+$ and $CD4^+$ immune responses were induced and AV-SLPs were presented to DCs more efficiently than the free SLPs. Moreover, T_H1 biased cytokines were induced effectively by AV-SLPs in patient-derived lymph node T cells. A phase I/II clinical trial is currently being conducted in HPV16 positive cancer patients to assess the safety and immunogenicity of AV-SLPs.[120] An additional example is a HIV vaccine comprised of large synthetic fragments from viral proteins featuring a covalently attached palmitoyl chain. This vaccine has achieved its goal in phase I/II trials, which evaluated its systemic safety as well as its capacity to elicit antigen specific CTL responses.[124]

Safety of Antigen Immunostimulant Conjugates. TLR agonists selected for covalent attachment are normally highly potent immunostimulants, inducing the secretion of high levels of proinflammatory cytokines and vasoactive substances, which may cause strong local inflammatory responses and contribute to the formation of systemic cytokine storm-like effects following administration.[125] Systemic dissemination of both antigens and TLR agonists is also observed to induce non-specific immune activation, which might lead to toxicity (e.g. proinflammatory cytokines) or undesired side effects (e.g. autoimmunity). Thus, when employed as adjuvants, TLR agonists may be associated with safety and tolerability concerns. Different strategies have been developed to tackle these issues, including: molecular modifications to produce TLR agonist derivatives that minimize systemic dissemination from their site of administration,[126] or improve their toxicity profile;[23,48] the development of candidates with limited biodistribution to minimize their off-site inflammatory impact (e.g. avoiding 'wasted inflammation');[127] optimizing formulations by incorporating TLR agonists into particulate carriers to control their release kinetics;[128] and conjugation of TLR agonists onto antigens.[129] Conjugating TLR agonists onto antigens enables efficient and precise delivery of both components to the same APC, which in turn reduces the required antigen and TLR agonist doses, thereby resulting in a decreased frequency of adverse effects. Thus, covalently attaching antigens with TLR agonists offers the possibility to dramatically reduce systemic exposure of these immunostimulants, but with sufficient vaccine components maintained at the local site during administration. Based on the results from clinical studies, concerns regarding the safety of antigen immunostimulant conjugate vaccines are unlikely to be of concern where doses have been evaluated, controlled and optimized. For example, influenza vaccines VAX 102/125/2012Q, all containing the TLR5 agonist Flagellin, cause no safety issues at the doses assessed in multiple phase I/II studies.[17-19] Trumenba®, which incorporates an N-terminal bacterial lipopeptide TLR2 agonist, was also well tolerated in human clinical trials.[68] Many other antigen-adjuvant conjugate vaccine candidates have also been demonstrated to be effective in animal models. Optimization of these conjugates to limit adjuvant related side effects and toxicity will be of high importance to ensure they are safe and well tolerated for human use.

CONCLUSION

Compared to traditional attenuated or inactivated vaccines, protein and peptide based vaccines are safer, easier to produce, and can elicit a more defined immune responses. However, protein or peptide antigens are normally poorly immunogenic, requiring adjuvants to improve their efficacy. Various adjuvants have proven to be effective at directing different types of adaptive immune responses. Among these adjuvants, TLR agonists are special since TLRs play a key role in regulating innate and adaptive immune responses to antigens. TLR agonists can activate DCs, facilitating the uptake of antigens and presentation of antigenic peptides in the context of MHC molecules. Adaptive immunity is then established through activation of antigen specific T lymphocytes and induction of specific humoral and cell mediated responses. Higher efficiency antigen presentation can be achieved where the antigen and TLR adjuvant are covalently attached together. Thus, developing subunit vaccine candidates with built-in TLR agonists has attracted wide attention in vaccine research and development. Different approaches, both chemical and biological, have been developed to produce protein or peptide based antigen-TLR ligand conjugates. However, the manufacturing of such conjugates is very sophisticated, and includes the chemical synthesis of peptide antigens or expression of protein antigens, the chemical synthesis or expression of TLR agonists, and their conjugation. Each step requires strict quality control to ensure that batch-to-bath quality is consistent and regulatory requirements are fully met. Most recently, the approval of Trumenba®, the first and the only commercial vaccine in this area, demonstrates the full potential of antigen-TLR ligand conjugate vaccines in extending the current vaccine pipeline and tackling unmet demands. This exciting achievement will potentially accelerate the development of various conjugation approaches with application to a number of different TLR agonists. Protein and peptide-based antigen-TLR ligand conjugate vaccines are on the cutting edge of vaccine research with intense ongoing research activities likely to lead to much more licensed products in the near future.

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Notes

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ABBREVIATIONS

TLR, Toll-like receptor; PRR, pattern recognition receptor; DC, dendritic cell; APC, antigen presenting cell; HPV, human papillomavirus; GAS, group A *streptococcus*; fHBP, factor H binding protein; SPPS, solid-phase peptide synthesis; MHC, major histocompatibility complex; PAMP, pathogen-associated molecular pattern; NK, natural killer; IL, interleukin; IFN, interferon; IgG, immunoglobulin G; CTL, cytotoxic T lymphocytes; GSK,

GlaxoSmithKline; FDA, Food and Drug Administration; LPS, lipopolysaccharide; HSV, herpes simplex virus; EBV, Epstein-Barr virus; LRR, leucine-rich repeat; MyD88, myeloid differentiation primary response factor 88; Pam3Cys, triacylated lipopeptide tripalmitoyl-S-glyceryl cysteine; Pam2Cys, diacylated lipopeptide dipalmitoyl-S-glyceryl cysteine; MALP-2, *M. fermentans* macrophage-activating lipopeptide; FSL-1, fibroblast-stimulating lipopeptide-1; MPLA, monophosphoryl lipid A; HSPCs, heat shock protein complexes; GMT, geometric mean titer; STF2, *Salmonella typhimurium* flagellin type 2; EDA, extra domain A from fibronectin; OVA, ovalbumin; NCL, native chemical ligation; AG, Arabinogalactan; OaAEP, *Oldenlandia affinis* asparaginyl endopeptidase; SrtAsa, *Staphylococcus aureus* sortase A; SLP, synthetic long peptide

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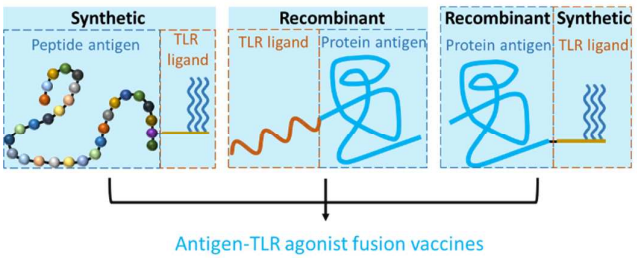
Table of Contents graphic (TOC)



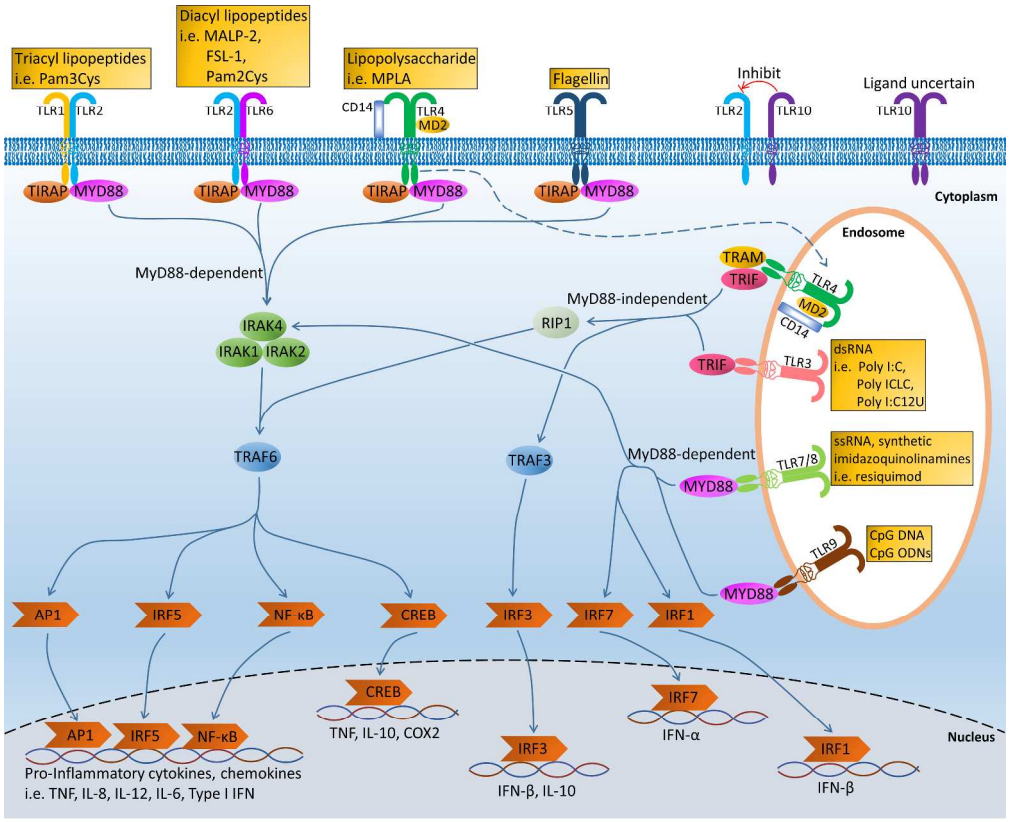


Figure 1. TLR signaling pathways of common TLR ligands. TLRs can detect exogenous PAMPs, resulting in the activation of TLR signaling pathways that lead to the regulation of transcription factors and the release of proinflammatory cytokines and chemokines. In the MyD88-dependent pathway, MyD88 is recruited to the cytoplasmic TIR domain to activate interleukin-1 receptor-associated kinase (IRAK) 4 and IRAK1/2, followed by activation of tumor necrosis factor receptor-associated factor 6 (TRAF6).43 Ultimately, this pathway leads to nuclear translocation of transcription factors including activator protein 1 (AP1) and nuclear factor-κB (NF-κB) which mediate induction of pro-inflammatory cytokines and chemokines such as TNF, IL-6, 8, 12 and type I interferon (IFN). The MyD88-dependent pathway also results in the translocation of cAMP response element-binding protein (CREB) which mediates induction of TNF, IL-10 and cyclooxygenase-2 (COX-2). For MyD88-dependent signaling TLR1, 2, 4 and 6 require the cooperation of TIRAP while TLR 7, 8 and 9 do not.42 TLR3 recruits TRIF to activate TRAF3 and thereafter interferon regulatory factor (IRF) 3 which results in the induction of IFN-β and IL-10. Unlike TLR3, TLR4 needs to firstly recruit TRAM before activating TRIF when signaling through the TRIF-dependent pathway. TLR7, TLR8 and TLR9 signaling leads to nuclear translocation of AP1 and NF-κB in MyD88-dependent pathway and activation of IRF7 and IRF1 that ultimately results in secretion of cytokines and chemokines such as TNF, IL-6, 12 and IFN-α, IFN-β. Overall TLR signaling results in host cell responses such as the induction of inflammatory mediators and production of antimicrobial agents. Many TLR agonists have been identified. For example, triacylated lipopeptide tripalmitoyl-S-glyceryl cysteine (Pam3Cys) as a TLR1/TLR2 heterodimer agonist;24,26 diacylated lipopeptide dipalmitoyl-S-glyceryl cysteine (Pam2Cys), M. fermentans macrophage-activating lipopeptide (MALP-2) and fibroblast-stimulating lipopeptide (FSL)-1 as TLR2/TLR6 heterodimer agonists;24 dsRNA and Poly I:C as TLR3 agonists;23 MPLA as a TLR4 agonist;25 bacterial heat shock protein complexes (HSPCs) including HSP70, HSP90 and gp96 possibly targeting TLR2 and TLR4;44-46 Bacterial flagellin as a TLR5 agonist;18 Imiquimod as a TLR7 agonist; ssRNA, gardiquimod, and resiquimod as TLR7/TLR8 heterodimer agonists; and CpG DNA and CpG ODNs as TLR9 agonists.21,22

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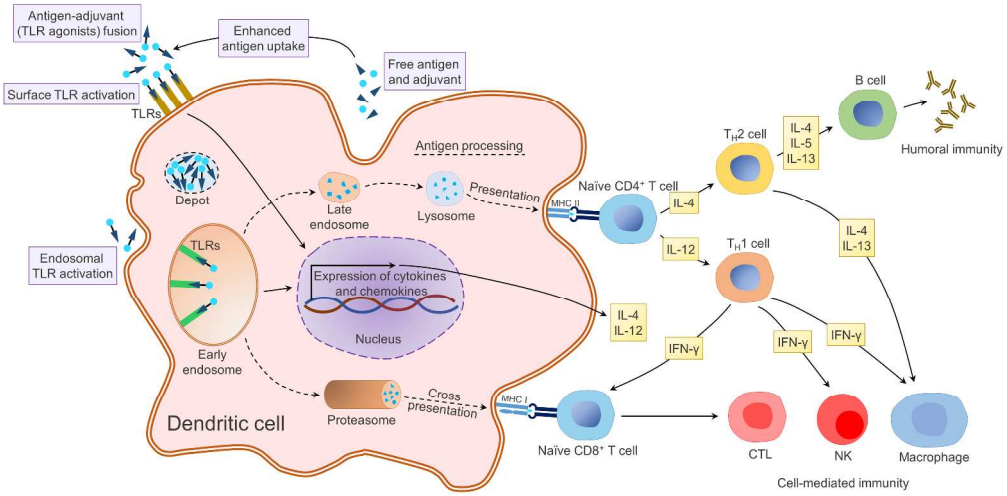

Figure 2. Putative mechanisms for the enhanced immunity associated with covalently attached TLR agonists. Administration of antigen-adjuvant fusions enables both antigen and TLR agonists to be delivered to the same DC at the same time, hence promoting efficient antigen presentation after conjugated antigens have been taken up by DCs. After internalization, TLR component helps to activate transcription factors that induce the production of cytokines and chemokines, such as IL-4 and IL-12, which help direct specific immune responses (e.g. TH1, TH2 and CTL type responses). Conjugated antigens are then processed in DCs, and their antigenic peptides are presented in the context of MHC class I or II molecules through presentation or cross presentation by DCs, which leads to the maturation of native CD4+ and CD8+ T cells.60-62 CD4+ T cells play a major role in shaping the adaptive immune system through: 1) promoting B cell maturation, which leads to the generation of memory B cells and long-lived plasma cells; 2) secreting different types of cytokines (e.g. IL-4, 5, 13 and IFN-γ), which play different roles in the development, maturation, and activation of macrophages and NK cells; and 3) activating CD8+ T cells and regulating CTL responses, which are central for host defenses against intracellular pathogens as well as the elimination of cancer cells.63-65

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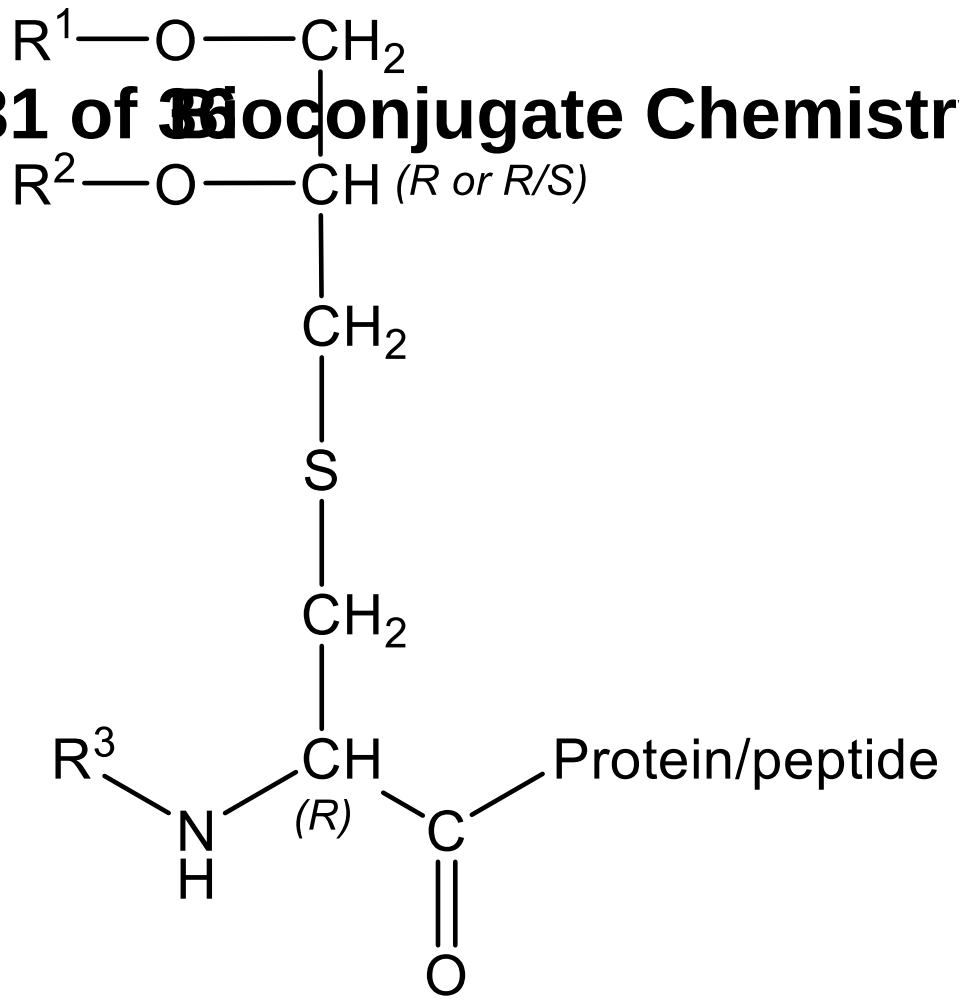

A Trumenba:

R^1, R^2=Mainly $CH_3(CH_2)_{14}CO$, or $CH_3(CH_2)_5CH=CH(CH_2)_7CO$, or $CH_3(CH_2)_5CH=CH(CH_2)_9CO$

R^3=$CH_3(CH_2)_{14}CO$

B Bexsero:

R^1, R^2=Mainly $CH_3(CH_2)_{14}CO$, or $CH_3(CH_2)_5CH=CH(CH_2)_8CO$, or $CH_3(CH_2)_{16}CO$, or $CH_3(CH_2)_6CH=CH(CH_2)_8CO$

R^3=H or $CH_3(CH_2)_{14}CO$

C Pam2Cys:

R^1, R^2=$CH_3(CH_2)_{14}CO$, R^3=H

D Pam3Cys:

R^1, R^2, R^3=$CH_3(CH_2)_{14}CO$

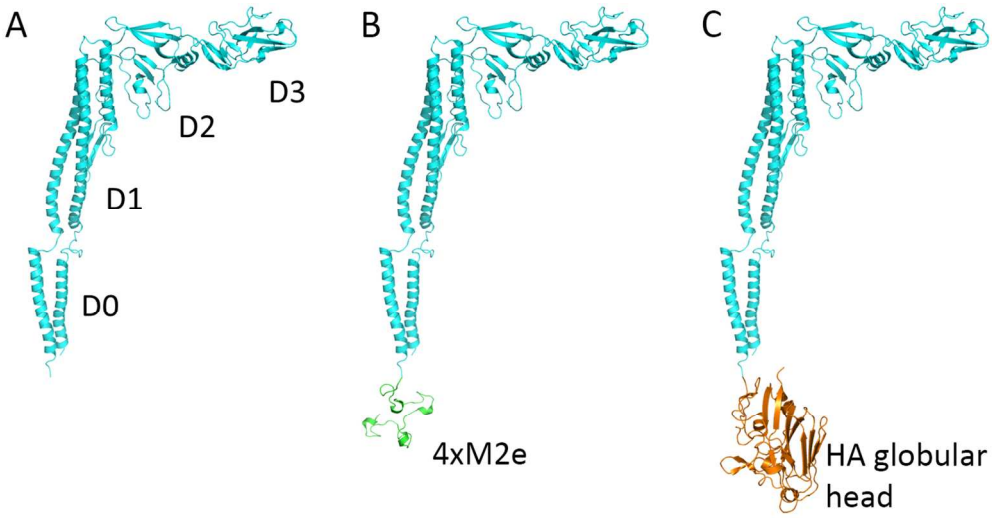

Figure 4. Schematic representation of flagellin and flagellin fusion vaccine candidates. (A) Flagellin. (B) VAX102, flagellin (cyan) with the C-terminus fused with four tandem copies of the ectodomain of the M2e (green). (C) VAX125, flagellin (cyan) with the C-terminus fused with a globular head of the HA1 domain (orange).

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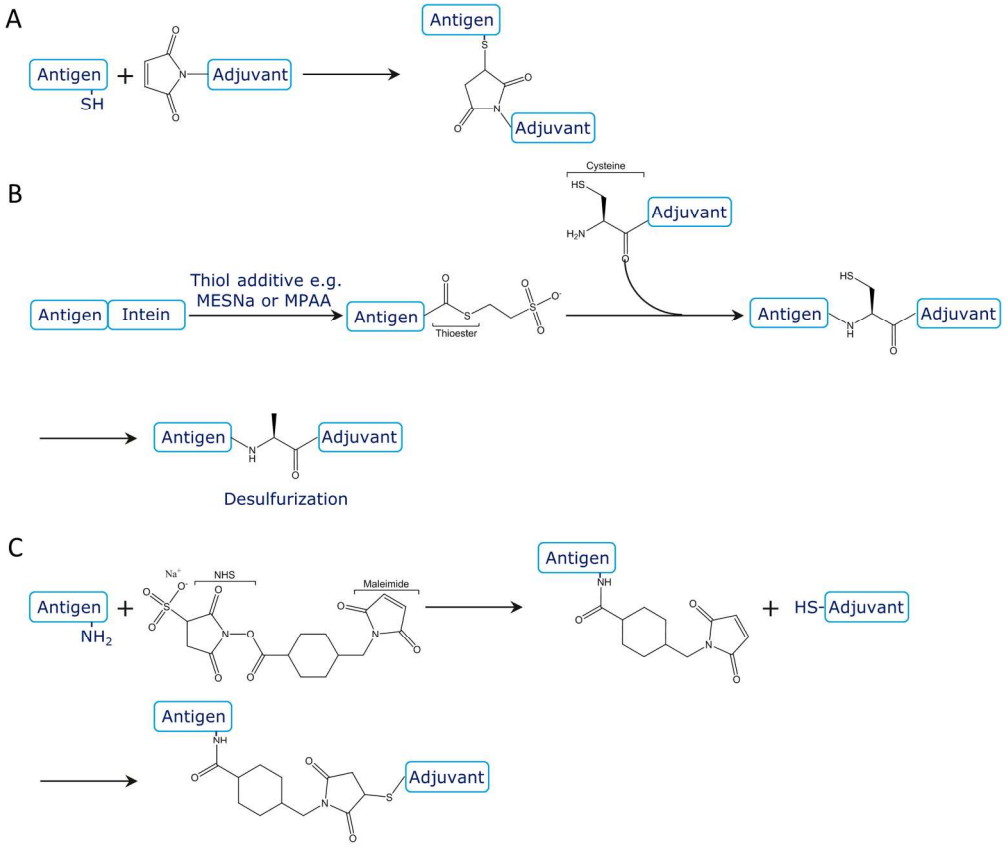

Figure 5. Examples of chemical ligation reactions suitable for the generation of antigen-adjuvant fusions. (A) Maleimide (thioether) ligation. (B) Expressed protein ligation. (C) Sulfo-SMCC (contains both NHS ester and maleimide group) was used in ligation reactions to conjugate the molecule of interest (contains a thiol group) onto antigen protein through lysine side-chains.

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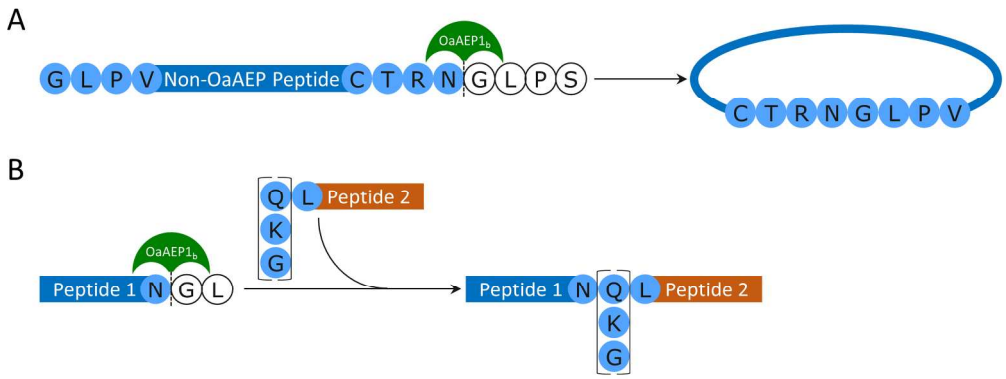

Figure 6. Schematic representation of OaAEP1b-mediated ligation. (A) Non-OaAEP peptides can be cyclized when fused to the recognition motifs present in the O. affinis cyclotide precursor. Peptide contains the C-terminal recognition sequence Cys-Thr-Arg-Asn-Gly-Leu-Pro-Ser and the N-terminal recognition sequence Gly-Leu-Pro-Val in this example. (B) For efficient ligation, C-terminal and N-terminal recognition sequences can be simplified to Asn-Gly-Leu and Gln/Lys/Gly-Leu, respectively.

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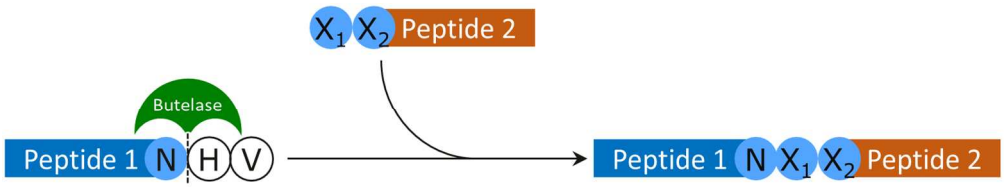

X_1= any amino acid except Pro

X_2= ILe/Leu/Val/Cys

Figure 7. Schematic representation of Butelase-mediated ligation.

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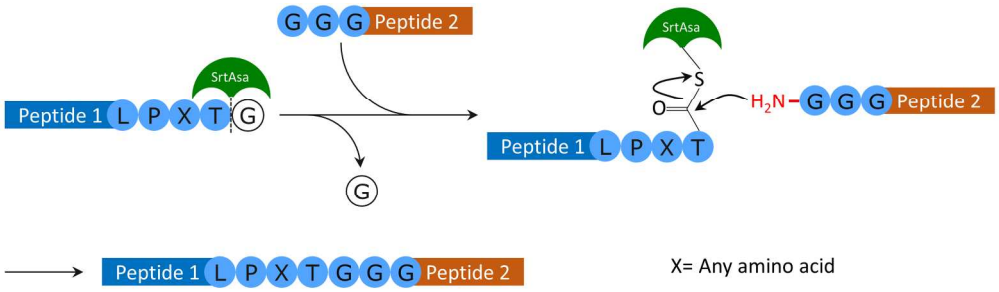

Figure 8. Schematic representation of SrtAsa-mediated ligation.

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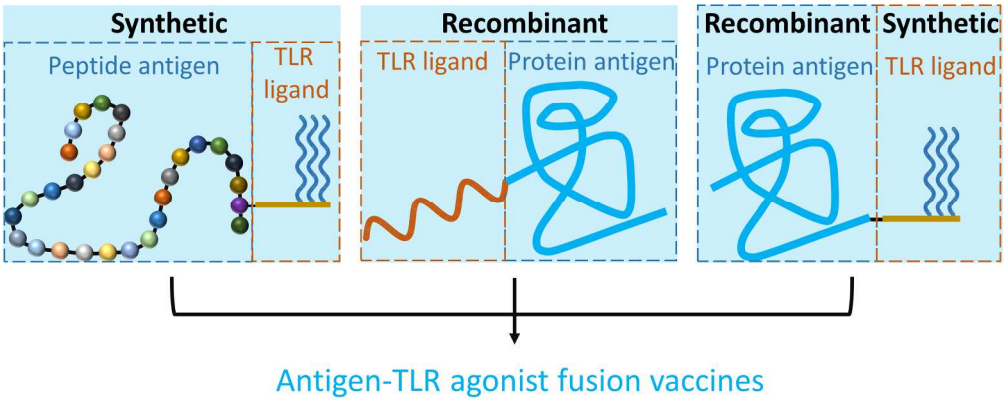

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