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Advances in the design and delivery of peptide subunit vaccines with a focus on Toll-like receptor agonists

Matthew Black,
University of California, Santa Barbara, CA 93106, USA, Tel.: +1 510 642 8977

Amanda Trent,
University of California, Santa Barbara, CA 93106, USA, Tel.: +1 510 642 8977

Matthew Tirrell, and
University of California, Santa Barbara, CA 93106, USA and University of California, Berkeley, CA 94720, USA, Tel.: +1 510 642 5833

Colleen Olive†
The Queensland Institute of Medical Research, PO Box Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia, Tel.: +61 073 362 0431, Fax: +61 073 845 3507

Matthew Black: mblack@engineering.ucsb.edu; Amanda Trent: trent@lifesci.ucsb.edu; Matthew Tirrell: mvtirrell@berkeley.edu; Colleen Olive: colleen.olive@qimr.edu.au

Abstract

Considerable success has been made with many peptide antigen formulations, and peptide-based vaccines are emerging as the next generation of prophylactic and remedial immunotherapy. However, finding an optimal platform balancing all of the requirements for an effective, specific and safe immune response remains a major challenge for many infectious and chronic diseases. This review outlines how peptide immunogenicity is influenced by the way in which peptides are presented to the immune system, underscoring the need for multifunctional delivery systems that couple antigen and adjuvant into a single construct. Particular attention is given to the ability of Toll-like receptor agonists to act as adjuvants. A survey of recent approaches to developing peptide antigen delivery systems is given, many of which incorporate Toll-like receptor agonists into the design.

Keywords

adjuvant; antigen delivery system; liposome; micelle; nanoparticle; peptide; subunit vaccine; Toll-like receptor; virus-like particle

Most common vaccine formulations contain inactivated pathogen, killed pathogen or pathogen-secreted toxins. While there has been great success using these vaccines for some

†Author for correspondence The Queensland Institute of Medical Research, PO Box Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia, Tel.: +61 073 362 0431, Fax: +61 073 845 3507, colleen.olive@qimr.edu.au.

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types of infectious diseases, there are many pathogens that are not amenable to such traditional approaches. Problems include inflammation and other unwanted host reactions, reversion to virulence, the inability to effectively culture the bacteria or virus, the possibility of inducing an autoimmune response, and the need for refrigerated storage [1–3]. For these reasons, there has been a shift towards developing subunit vaccines that contain short, specific fragments of a pathogen that are noninfectious because they lack any potential to replicate in the host. This rational approach to vaccine design aims to create vaccines that are safer and more immunologically defined compared with the traditional empirical approaches.

Synthetic peptides have attracted considerable attention as a basis for subunit vaccine design. Peptides play critical roles in determining the magnitude and specificity of cell-mediated (T cells) and humoral (antibody) immunity, and can contain the minimal sequences necessary for immunomodulation. Delivered correctly, peptides harboring B- or T-cell receptor epitopes are sufficient to elicit an effective immune response. In addition to stimulating a protective response against a particular pathogen, peptide-based immunotherapy can also be used to treat other chronic ailments such as cancer, or be developed for immune suppression for autoimmune diseases. Furthermore, peptides can be easily synthesized and characterized, and are generally more stable than whole pathogens or full-length proteins.

Although they are promising candidates as the next generation of immunotherapeutics, peptides are typically poorly immunogenic when used alone as vaccines. They rely on delivery with potent immunostimulatory adjuvants to effectively activate the innate and adaptive arms of the immune system. Many types of vaccine adjuvants have become available since the 1970s, but to date only a few have been approved for human use, consequently slowing the development of synthetic peptide vaccines [4]. Significant advances in the engineering of adjuvants in the past decade, such as Toll-like receptor (TLR) agonists and novel particulate delivery systems, have made peptide-based vaccines a viable option for modern immunotherapy. This is evidenced by the recent surge in publication of preclinical studies, as well as by the numerous human trials that are currently underway. This review will examine the broad range of peptide antigen delivery systems presented in current literature, with a focus on using TLR agonists as adjuvants.

Design considerations for peptide antigen delivery systems

Understanding the role peptides play in immunity and how different peptide properties can affect how the immune system recognizes and responds to them is important when developing strategies for peptide-based immunotherapy. Ultimately, the way in which peptides are presented to the immune system will determine how or if the peptides are recognized and processed, and consequently whether or not an appropriate immune response will follow [5].

Properties affecting B-cell stimulation

The specific properties of different peptide antigens can have profound effects on immune recognition and the ensuing response. For example, there is evidence suggesting that the ordered, highly repetitive display of antigens, as seen with bacterial polysaccharides and viral glycoproteins, may be an important trigger for a humoral response [6]. Specifically, the multivalent presentation of antigens can induce the proliferation of antibody-producing B cells independent of T-cell help by cross-linking adjacent membrane-bound immunoglobulin molecules on the B-cell surface (i.e., antigen-specific B-cell receptors). The activated B cells then rapidly secrete pentameric antibodies (compensating for low antigen affinity) to fight against initial infection, allowing time for antibody affinity maturation, T-cell recruitment and memory cell development. It has also been reported, however, that a second signal is needed to stimulate the secretion of antibodies by activated B cells, presumably to confirm that the antigen is foreign and not self-derived [7].

Understanding the physical parameters influencing B-cell receptor cross-linking and activation can be valuable for rationally engineering peptide vaccines, particularly those designed to elicit a strong humoral response. For example, the average distance between the binding sites of adjacent receptors is calculated to be 35 nm [6], the distance between repetitive epitopes on a number of antigens capable of cross-linking B-cell receptors is shown to be on average 5–10 nm [8,9], it is estimated that 10–20 receptors need to be cross-linked per cluster, and only a small number of clusters are necessary for B-cell proliferation [9,10]. Further investigation into the level of order and rigidity of multivalent antigens would be beneficial for subunit vaccine design. Nevertheless, an antigen delivery system that can present multiple copies of an antigenic peptide in a single complex, thus increasing the local antigen concentration and providing multivalency, has the potential to generate a significant antigen-specific immune response. Delivery systems capable of multivalent peptide display have indeed been developed, including the dendrimeric multiple antigen peptide system first introduced by Tam and colleagues and then built upon by others [11–14], as well as a number of other creative and more recent designs that will be discussed later in this review.

Conformational integrity is another cause for weak immunogenicity. Peptides taken out of their native environment within the protein typically do not contain enough structural information to fold correctly. In regard to stimulation of an antibody response, this is important because B-cells recognize both the specific sequence and the specific shape of their target. If the peptide epitope does not share a similar structure to that assumed by the same sequence in the target protein, the resulting antibodies will probably not cross-react with that protein. Consequently, unless the desired epitope falls within an unstructured region of the protein (e.g., loops or turns), peptide-based vaccines must promote the peptide secondary structure in order to induce an antigen-specific humoral response. There are many sequence-based algorithms developed to predict B-cell epitopes; however, even in combination, they are not particularly accurate. More recent methods that take into account protein 3D structure have had more success, but they are still far from being able to reliably predict exact B-cell epitopes [15,16].

Approaches to mimicking native peptide structure have proved successful, as illustrated with trials using the conformation-dependent epitope found in the coiled-coil α-helical M protein of the group A streptococcus (GAS) bacterium [17,18]. Synthetic peptides representing a protective B-cell epitope within a GAS M protein conserved region did not possess the correct helical structure for B-cell epitope integrity. However, when flanked by other peptide sequences that possessed a propensity for folding into an α-helix, the resulting antibodies were opsonic and protected mice against GAS infection. Enhancement of peptide secondary structure has also been demonstrated in protein analogous micelles. These micelles self-assemble from peptide amphiphiles, which consist of a hydrophilic peptide conjugated to a hydrophobic alkyl tail. Upon self-assembly, it has been shown that short collagen peptides regain their triple-helical structure [19,20] and that a DNA-binding peptide regains its α-helical structure [21]. Other approaches to enhancing antigen structure involve using peptide scaffolds known as template-associated synthetic proteins and sequential oligopeptide carriers [13].

Properties affecting T-cell stimulation

Rather than recognizing native, intact antigen as B cells do, T cells specifically recognize processed antigen fragments bound to MHC molecules located on the surface of antigen-presenting cells (APCs). For this reason, peptide conformation is not a critical factor when stimulating T cells. Other properties, such as peptide length and peptide–MHC interactions, have been studied to aid in the identification of T-cell epitopes [5,22]. MHC class I molecules, which display peptides to cytotoxic T cells (T_c cells), and MHC class II molecules, which display peptides to helper T cells (Th cells), both possess an antigen-binding cleft composed of an antiparallel β-sheet floor bound by two α-helices. The cleft is lined with pockets of highly

polymorphic amino acids that serve as the basis for antigen restriction. In MHC-I molecules, conserved residues residing on each end of the binding cleft are involved in hydrogen-bonding interactions with the termini of bound peptide antigens, effectively limiting the length of the antigens to approximately 8–11 amino acids. MHC-II molecules lack these hydrogen-bonding residues and consequently allow the antigen to protrude from the ends of the cleft. Peptides bound to MHC-II molecules tend to be approximately 13 amino acids in length, but can be considerably longer [5].

Interestingly, it has been shown that minimal T_c-cell peptide epitopes can be loaded onto MHC-I molecules present on the surface of APCs without undergoing natural intracellular processing [23]. Simply using minimal length T-cell peptide epitopes, however, is not necessarily the best strategy. Longer peptides that must be taken up by APCs will undergo processing where they are cleaved, loaded onto appropriate MHC molecules and transported to the cell surface, ensuring proper presentation of the peptides. Epitopes incorporated into longer peptides [24], such as those containing repeating or multiple epitopes, or flanked by nonepitope sequences [25], have been shown to be effective in stimulating an immune response. There are several prediction algorithms available that attempt to predict what peptide sequences will fit into each of the MHC molecule types [22,26,27]. These bioinformatics tools are generally more successful than those used to predict B-cell epitopes, but they are still not totally accurate. *In vitro* tests that aim to rapidly and easily identify appropriate epitopes, including high-throughput phage display [28] and micro-arrays [29], have also been developed. These methods can be used in combination for greater accuracy, but the resulting peptides still need to be verified *in vivo* to ensure they are able to create an immune response.

Recruiting Th cells

A robust immune response often requires stimulation of Th cells. Activated Th cells recruit other naive Th cells, T_c cells and B cells, providing signals necessary to amplify the immune response against the target antigen. Th cells also initiate the development of memory B and T cells to provide long-lasting immunity against the antigen upon subsequent exposure. Small, synthetic peptides designed to induce a humoral or cell-mediated immune response usually do not contain an appropriate Th-cell epitope and therefore are not always effective vaccines by themselves. Adding further constraint to Th-cell stimulation is the high degree of MHC polymorphism. Incorporating a promiscuous Th-cell epitope that binds to multiple MHC-II alleles into a peptide antigen delivery system represents a feasible solution to these problems.

A traditional method of strengthening the immunogenicity of a peptide antigen is to covalently attach it to a large carrier protein, such as keyhole limpet hemocyanin [30,31] or diphtheria toxoid [32], which provides a source of Th-cell epitopes. While using a carrier approach can lead to the production of an immune response against the peptide antigen, there are a number of drawbacks. The carrier protein can itself elicit an immune response, competing with or even suppressing the response towards the peptide antigen [33,34]. An antibody of irrelevant specificity may also be induced by constructing and attaching the peptides in the incorrect order and orientation. Other limitations include the amount of peptide that can be loaded onto the carrier, the dose of carrier protein that can be safely administered and the relatively low quality control that can be achieved [5].

To circumvent these problems, target peptide antigens have been conjugated or combined with other short peptide sequences containing defined Th-cell epitopes. This has been shown *in vitro* and in animal studies to provide heightened immune responses towards target peptides with little or no evidence of immune suppression [5,35,36]. For example, a synthetic peptide vaccine based on luteinizing hormone-releasing hormone (LHRH), which plays a central role in the reproductive process, and promiscuous Th-cell epitopes were formulated in an oil-based emulsion or adsorbed onto an aluminum hydroxide gel. The vaccine was immunogenic in

rodents, dogs and a nonhuman primate, baboons. The antibody response was specific to the LHRH peptide in contrast to conjugating the LHRH peptide to a carrier protein, where only a small portion of the immune response was directed at the peptide [37]. It should be noted, however, that if the peptides are linked, there is also the potential for antibody generation against epitopes formed at the junction of the T-cell and B-cell epitope-containing peptides.

Adjuvants & the innate immune system

Even with the optimal B- or T_c-cell epitopes and appropriate Th-cell epitopes, peptide-based immunotherapeutics still require adjuvants to be effective [38]. An adjuvant is broadly defined as anything that can increase the immune-stimulatory behavior of an antigen. Adjuvants function in a number of ways, including providing an antigen depot for increased uptake by APCs, shielding peptides from rapid degradation, increasing the peptide half-life and/or by directly activating pattern-recognition receptors (PRRs) on APCs [39,40]. All of these mechanisms involve stimulating APCs directly or indirectly, especially dendritic cells (DCs) [41].

Dendritic cells provide a crucial link between the innate and the adaptive immune system by nonspecifically internalizing and processing antigen, and presenting the resulting antigenic peptides to sequence-specific T cells. When the PRRs on DCs are stimulated, immature DCs participate in the innate immune response by releasing various soluble mediators, such as inflammatory cytokines and type I interferon (IFN) [42]. Immature DCs reside in both peripheral tissues and in the lymphoid tissues [42]. DCs that reside in the peripheral tissues migrate to regional lymph nodes upon activation. After activation, DCs initiate an adaptive and antigen-specific immune response by processing antigens and presenting them to naive T cells in the lymph nodes [43]. At the same time, DCs upregulate MHC class II and costimulatory molecules, promoting interactions between DCs and T cells [44]. Stimulating DCs shapes adaptive immunity by controlling the type, quality and magnitude of the immune response and memory. The balance between inflammatory and anti-inflammatory cytokine production in DCs, such as IL-12 and IL-10, respectively, is crucial in determining the type of Th-cell response. For example, IL-12 drives polarization of Th cells towards a Th1 response, which favors stimulating T_c cells, and mediates immunity to most pathogens [45], notably intracellular pathogens [46,47]. IL-10, on the other hand, has a regulatory role and can mediate a Th2 response, which leads to humoral immunity and anti-inflammatory pathways [48,49].

Aluminum hydroxide or phosphate salts, commonly called alum, have dominated adjuvant use since the discovery of alum in the 1920s (as used with diphtheria, tetanus and hepatitis B vaccines) [50]. The mechanism of action of alum has been debated, but recent research suggests alum functions by activating DCs [51] through a PRR known as the NALP3 inflammasome [52] in addition to providing a depot for the antigen [53]. Alum, while often effective at generating humoral immunity, is ineffective at inducing cell-mediated immunity [53]. Furthermore, aluminum-based adjuvants have in some cases been associated with serious local reactions, such as erythema, subcutaneous nodules and contact hypersensitivity [54,55]. In order to avoid these limitations of alum, there is a growing need for new adjuvant formulations.

Many adjuvants, such as emulsions, function in a manner similar to alum by providing a peptide antigen depot. Antigen depots enhance peptide immunogenicity by concentrating the peptides and extending the time antigen resides in the body, thus increasing the probability of interaction with immune cells [56]. Many antigen depots are also theorized to create inflammation and stimulate the recruitment of APCs [57]. MF59, an oil-in-water emulsion, is an approved adjuvant in Europe that is theorized to primarily function in this manner [4]. Creating an antigen depot for peptide vaccine design remains a useful strategy, as is illustrated by the Phase II clinical trial showing successful treatment of stage 3 vulvar neoplasia using multiple human papillomavirus (HPV)-16 peptides emulsified in incomplete Freund's adjuvant [58].

Incomplete Freund's adjuvant is a water-in-oil emulsion that is now in Phase III clinical trials [4].

Antigen depots are effective in many situations. However, as knowledge of the immune system has grown, much of the recent vaccine adjuvant research is aimed at specifically activating DCs by targeting PRRs. PRRs are germ-line encoded and recognize distinct evolutionarily conserved microbial structures called pathogen-associated molecular patterns (PAMPs). TLRs, cytosolic receptors from the nucleotide-binding oligomerization domain-like receptor family and the retinoic-acid-inducible gene I-like receptor, the NALP3 inflammasome, lectin receptors and mannose receptors, are all types of PRRs [59]. While all of the PRRs may be suitable targets for vaccine adjuvants, TLRs have been particularly well characterized [60]. TLRs activate DCs and produce primarily a Th1 immune response [61,62], although Th2 responses can be elicited in some instances [63,64]. Using TLR agonists as adjuvants has been shown to produce more and higher avidity T-cell clones than alum [65]. Different TLR agonists in conjunction with a model antigen for immunization of non-human primates have also been shown to influence the magnitude and quality of memory T-cell responses [66]. TLR agonists can be used in novel adjuvant formulations, or combined with more traditional adjuvants, such as alum or emulsions, to improve their efficacy. The safety of TLR agonists has also been established in clinical trials [67,68] and the TLR4 agonist monophosphoryl lipid A (MPLA) has been approved for human use in Europe [69] and, in late 2009, in the USA, was coformulated with an alum called AS04.

TLR agonists

Humans have ten functional TLRs (TLRs 1–10) [41]. The TLRs are highly conserved and, while different organisms have varying numbers of TLRs, most vertebrates contain at least one TLR from each of the six major TLR families [70]. Humans, notably, have functional members from only five of the TLR families, containing only a pseudogene for TLR11. Mice, in which the majority of experiments determining TLR function have been carried out, have the same TLRs as humans, with the exception of TLR10, and contain three additional functional TLRs, TLRs 11–13 [71].

Toll-like receptors function as either homodimers [72] or heterodimers with other TLRs [73]. Some TLRs require the help of other proteins, notably TLR4 with MD2 [74]. TLRs 1, 2, 4, 5, 6 and 10 are all expressed on the cell surface and migrate to phagosomes after activation. TLRs 3, 7, 8 and 9, all of which recognize nucleic acid motifs, are confined to endosomal compartments [75].

All of the TLRs recognize common conserved motifs (i.e., PAMPs) in bacteria, viruses, fungi or other pathogens (Table 1). Ligands for TLRs 1–9 have been identified, but not for TLR10 [76]. TLR2 forms dimers with TLR1 and TLR6 to recognize various lipopeptides, TLR4 recognizes bacterially associated lipopolysaccharide (LPS) and TLR9 recognizes CpG DNA and unmethylated CpG-containing oligodinucleotides (ODNs). In addition to natural ligands, synthetic ligands have been developed for many of the TLRs. Several synthetic imidazoquinoline-like molecules have been developed for TLR7 and TLR8, and MPLA has been developed as a TLR4 agonist that is significantly less toxic than LPS [77–80].

Toll-like receptors are expressed on a wide variety of cells, including B cells, specific types of T cells, monocytes, DCs, macrophages, certain epithelial cells and many other innate immune cells [81]. Expression patterns on the different cells vary. Different types of DCs also have different expression patterns. Plasmacytoid DCs, which are critical in responding to viral nucleic acids, express TLR7 and TLR9 [82], myeloid DCs express TLRs 1, 2, 3, 5, 6 and 8, and human monocytes express TLRs 1, 2, 4 and 5 [83].

TLR signaling

While early vaccine adjuvants were approved without the exact mechanism of action being known, understanding the way that new adjuvants function will be necessary for their approval and will aid in their design. Upon binding to the respective ligands, TLRs initiate complex intracellular signaling pathways that result in the creation of an inflammatory environment and an adaptive immune response (Figure 1). TLR signaling has been reviewed in detail [84–86] and will only be briefly discussed here. TLRs are type 1 transmembrane proteins composed of an extracellular domain containing leucine-rich repeats involved in the recognition of PAMPs and a cytoplasmic Toll/IL-1 receptor (TIR) domain, which recruits various TIR-containing adapter molecules and initiates the intracellular signaling cascade.

Two independent pathways have been distinguished for TLR signaling. The myeloid differentiation primary-response gene 88 (MyD88)-dependent pathway can be activated upon engagement by all TLRs except for TLR3. MyD88 binds TLRs directly through their TIR domain or, for TLR4, through the MyD88 adapter-like TIR domain-containing adapter protein [87]. For TLR2, it has been shown that MyD88 adapter-like TIR domain-containing adapter protein is only required at low ligand concentrations [88]. MyD88 recruitment then leads to the activation of the IL-1 receptor-associated kinase signaling molecules [89], which eventually activate several MAPKs and key transcription factors, including NF-κB, activator protein 1, and IFN regulatory factor 3/7, leading to the secretion of cytokines and DC maturation [84].

The MyD88-independent or TIR-domain-containing adapter inducing IFN-β (TRIF)-dependent pathway can be stimulated by TLR3 and TLR4. It involves IFN regulatory factor 3-mediated IFN-β production, activation of signal transducer and activator of transcription 1 and 2, and stimulation of type I IFN-inducible genes [82]. The TRIF-related adapter molecule is important in the MyD88-independent signaling mediated by TLR4 [90]. Stimulation of TLR signaling via the TRIF-dependent pathway also leads to activation of NF-κB and MAPK signaling pathways [84].

TLR synergy

Most pathogens present multiple TLR agonists to the immune system. It has been shown that TLRs cooperate in resistance to infectious diseases [91,92]. Several groups have shown that stimulating multiple TLRs results in a synergistic upregulation of cytokines [93–96]. In other words, the effect that two or more TLR agonists have on the immune system is much greater than any single agonist. In a comprehensive evaluation of different TLR agonists, DCs were shown to exhibit a synergistic upregulation of inflammatory cytokines when stimulated with several, but not all, TLR agonist combinations, notably TLR3 or TLR4 combined with TLR7, 8 or 9 [97]. It has been suggested that TLR agonists that signal through the MyD88- and TRIF-dependent pathways cooperate in TLR synergy [93–95]. Warger *et al.* showed that the synergy through the MyD88 and TRIF pathways of peptide-loaded DCs led to a marked increase in T_c-cell effector function in wild-type mice *in vivo* [98]. TLR agonists have also been shown to act synergistically with other PRRs [99–102]. There is much room for exploration on what combinations of agonists will work best in all cases. However, stimulating multiple PRRs, specifically TLRs, can be a powerful tool for inducing a strong immune response and could be beneficial in many vaccine formulations.

Risks with TLR agonists

Activating TLRs is not without risk. Toxicity and safety are concerns given the strong inflammatory responses stimulated by TLR agonists. TLRs are involved in autoimmune, chronic inflammatory and infectious diseases [103,104]. Sepsis, perhaps the most serious, is caused by LPS overstimulating TLR4, resulting in organ failure [105]. TLRs have also been implicated in diabetes [106] and atherosclerosis [107]. While negative regulatory pathways

exist [108], they can be difficult to control in a vaccine setting. TLR9 agonists have been shown to be able to break tolerance and cause autoimmune disease in animal models [109]. Repeated exposure to single TLR agonists has been shown to induce T- and natural killer cell immunosuppression [110]. In fact, certain viruses have been shown to utilize TLR2-induced immunosuppression to avoid immune recognition [111].

It has also been shown that stimulating TLRs is not necessary to generate an antibody response and suggested that vaccine strategies avoiding this may still be able to stimulate the immune system, and would avoid the risks associated with TLR stimulation [112]. However, it has been demonstrated that TLR agonists can significantly enhance vaccine efficacy and lead to the appropriate cellular and antibody responses. The concerns of using TLR agonists highlight the need for effective delivery vehicles that minimize the risks associated with them while maximizing their benefits.

Multifunctional peptide antigen delivery systems

Delivering antigenic peptides with TLR agonists is a promising direction for the development of new immunotherapeutics. However, determining what type of adjuvant and which peptide epitopes to include in the design is only part of the story. As outlined above, immunogenicity is dependent on how the peptide antigen and adjuvant are recognized by the immune system. The route of administration and the specific vaccine formulation will have a profound effect on factors such as peptide orientation and structure, stability of peptides against degradation and clearance, tissue localization, antigen uptake and processing, and toxicity. All factors considered, optimal peptide-based vaccines should be presented in a multifunctional delivery system that can address these issues and promote a safe and robust immune response. Some of the most promising approaches taken to deliver peptide antigen and adjuvant together include covalently linking peptides to TLR agonists and delivering peptide and adjuvant together in novel particulate carriers (Figure 2).

Antigen–adjuvant conjugates

For an effective immune response against the peptide antigen, the same APC should process the antigen and at the same time be stimulated by the adjuvant [113]. In fact, it has been shown that the antigen and TLR agonist should colocalize to the same phagosome for efficient MHC-II antigen presentation [114]. Covalently linking the TLR agonist to the peptide antigen is perhaps the most straightforward way to ensure that the same APC will encounter both the antigen and adjuvant. Lipopeptides have proven to be effective, synthetic ‘self-adjuvanting’ vaccines, which also function, at least in part, by stimulating TLR2 [115,116]. Importantly, enhanced peptide immunogenicity is seen only when the peptide and lipid are coupled and not when simply mixed together [117]. Furthermore, it has been demonstrated that the linker location, linker chemistry and the number of lipid groups can significantly influence the immune response [118,119]. It should be noted that an added advantage of these amphiphilic constructs is that they can act as modular building blocks in self-assembled structures, such as liposomes and micelles [120].

Many peptide antigen–lipid conjugates have been explored. In a simple design, attaching a single palmitic acid moiety to an antigenic peptide has been shown to enhance peptide immunogenicity in a TLR2-dependent manner [121]. Jackson and coworkers reported that conjugating antigenic peptides to dipalmitoyl-S-glyceryl cysteine (Pam_2Cys) has generated antigen-specific immune responses in a wide variety of animal studies. These studies include those evaluating B-cell epitopes for a contraceptive therapy [117], and T_c-cell epitopes for hepatitis C virus [122], influenza virus, intracellular bacterium *Listeria monocytogenes*, and the model tumor antigen ovalbumin [123]. In another sophisticated design, Olive and coworkers have shown that lipid-core peptides, which link synthetic analogs of Pam_3Cys (i.e.,

lipoamino acids) to multiple peptides via dendrimeric multiple antigen peptide structures, are effective for GAS vaccines [124,125]. This system allows for the delivery of multiple peptides in one complex, making it possible to include different epitopes that may be needed to achieve broad-spectrum immunity [126]. In addition to stimulating TLR2, lipid-core peptides and other lipopeptides have been reported to cross mucosal surfaces, allowing for needle-free vaccines capable of inducing IgA-mediated mucosal immunity [127,128]. Clinical trials have demonstrated the safety and efficacy of lipopeptide vaccines, including those against HIV [129] and HPV [130].

Antigens have also been coupled with other TLR agonists. It has been shown that covalently linking HIV-1 gag protein to a synthetic TLR7/8 ligand (an imidazoquinoline-like molecule) was effective at generating HIV-1 gag-specific T_c cells, while simply codelivering them did not induce a strong response [131]. In another example, CpG–antigen conjugates were shown to be more effective than codelivery, and even competed with live virus vaccines due to enhanced antigen cross-presentation and stimulation of DCs [132]. Supporting these results, Khan and colleagues demonstrated that linking either Pam_3Cys or CpG to peptide antigens increased antigen uptake and cross-presentation by DCs, although the uptake mechanisms were different [133]. Covalently linking TLR agonists with peptides is not the only option to couple the two. For example, a complex between positively charged HPV-16 E7 peptides and negatively charged poly(I:C) synthetic dsRNA, a TLR3 agonist, was capable of effectively stimulating tumor regression in a human cervical cancer model in which tumors were pre-established in mice [134].

Particulate delivery systems

Particulate delivery systems have shown promise in a wide variety of fields for incorporating multiple functions into one particle. For example, modular micelles that self-assemble from peptide amphiphiles have been developed to target tumors [135] and deliver therapeutic peptides to atherosclerotic plaques *in vivo* in mice [136]. Similarly, modular multifunctional vaccine platforms can incorporate peptide epitopes with adjuvants and be targeted to the appropriate immune cells. While the specific components of each multifunctional carrier vary widely, a common theme is that they form some type of nano- or micro-scale particulate. It has been shown that the carrier can often exhibit self-adjuvanting behavior. Being that bacteria and viruses can also be roughly defined as particulates of similar size, it is suggested that the particulate design in general could act as another type of PAMP and impart enhanced immunogenicity [137,138].

The precise size of a vaccine particle can influence its bio-distribution and the mechanism of particle uptake, and thus influence the type or level of the resulting immune response. Large particles (>0.5 μm) are primarily taken up by macrophages via phagocytosis, whereas microparticles ranging from 0.5 to 5 μm in size are predominantly taken up by macropinocytosis. Virus-sized particles with diameters of approximately 20–200 nm are taken up by DCs [139] through endocytosis via clathrin-coated pits, caveolae or specific receptors [140].

Understanding that size may be a critical factor in vaccine design, Plebanski and colleagues examined the uptake and immunogenicity of peptide antigen-coated beads ranging from 20 to 2000 nm in diameter. They found that 40–50 nm particles were preferentially taken up by DCs and promoted a significantly stronger cell-mediated response than larger particles (>0.5 μm), while the larger particles were mainly taken up by macrophages and were capable of inducing a considerable antibody response [139]. A later study showed that the same peptide-coated beads within the narrow size range of 20–123 nm could differentially affect cytokine production [141]. Moreover, it is has been shown that particles less than 50 nm in diameter are efficiently taken up into lymphatic vessels by the interstitial flow and transported to regional

draining lymph nodes where there are concentrated populations of DCs, while particles greater than 100 nm generally remain near the site of administration [39,142–144]. It is not obvious that one specific size range is optimal for all vaccine formulations, but it is clear that controlling the size of a vaccine particle could be a means to bias the immune response.

Aside from size, a multitude of other functionalities can be engineered into particulate-based vaccines. Particles can display a multivalent array of peptides that can potentially cross-link B-cell receptors to initiate a strong antibody response [145]. Particulate-based vaccines can also encapsulate peptides to protect them from degradation and act as a depot for antigen release over time. Cell-specific ligands can be integrated on particles for active targeting and additional adjuvants can be incorporated to amplify or direct the immune response. A large number of particulate delivery systems encompassing a wide range of sizes (nm to μm) and materials (natural and synthetic) have been developed for peptide-based immunotherapy. In many cases, the immunostimulatory properties of the particles have been significantly enhanced through the addition of TLR agonists.

Lipid-based carriers—Liposomes are a versatile platform for the construction of multifunctional peptide antigen carriers. Liposomes consist of a lipid bilayer composed of natural or synthetic phospholipids that surround an aqueous core. Liposomes can be made as large multilamellar structures several microns in diameter or, with the correct lipid composition, can be made any range of sizes down to approximately 30 nm by using techniques such as sonication or extrusion. Peptide antigens can be conjugated to lipids and displayed on the liposome membrane [146,147] or be encapsulated in the liposome core [148]. The lipid used to anchor the peptide to the liposome can itself be a TLR2 agonist [147]. Additional TLR agonists, such as MPLA, can also be incorporated into the liposome bilayer [149]. Clinical trials with lipopeptides and MPLA in the liposome bilayer have been conducted and proved to be safe and effective for non-small-cell lung cancer [149,150]. To incorporate other TLRs, negatively charged ODNs, such as TLR3 and TLR9 agonists, can be complexed with positively charged lipids on the liposomes for delivery [151–153]. Small molecules, such as synthetic TLR7/8 agonists, can be encapsulated in the liposome core [154].

It has been reported that combining TLR agonists with peptide antigens functions optimally only when the peptide antigen and TLR agonist are presented on or in the same liposome [155]. In addition to simultaneously delivering TLR agonist and peptide antigen, liposomes can also serve to protect the TLR agonist from degradation. This is particularly important with regard to ODNs, which are vulnerable to the abundant nucleases in the body [156,157]. Liposomes can also be used to target both peptide and antigen to DCs. They may target DCs passively by being preferentially taken up by DCs based on their particulate nature. Attaching active targeting ligands, such as mannosylated lipids [158] or the DEC-205 antibody [159] have also been shown to increase the efficacy of liposomal antigen carriers. Liposomes are also capable of targeting their contents to endosomal TLRs [160] and antigen to the MHC pathways [161].

The composition of the liposome is an important variable. It has been shown that increasing the membrane fluidity can enhance antibody responses [162,163]. Taneichi *et al.* showed that antigen coupled to liposomes using saturated, but not unsaturated, fatty acids induced a Th1 response to ovalbumin peptide [164]. This was shown to be because the saturated fatty acids with antigen were processed and peptides displayed by MHC class I molecules, while peptides derived from antigen linked to unsaturated fatty acids were only displayed through the MHC class II pathway. While many liposomes are considered to be more or less inert carriers that protect antigen from degradation and deliver antigen to APCs, the lipids themselves can function as adjuvants in the absence of other immunostimulatory molecules. These include liposomes formed from different bacterial lipid extracts [165,166], which may actually

stimulate TLRs, and various combinations of cationic lipids [167,168]. However, many liposomes have been shown to need an additional adjuvant in order to be effective [169].

Another lipid-based antigen delivery strategy is to form immunostimulatory complexes in which the peptide antigens are embedded in hollow, 40-nm diameter particles comprising of phospholipids, cholesterol and the plant-derived immunostimulatory saponin Quil A. ISCOMATRIX™ (CSL Behring, PA, USA) vaccines, which are made by mixing peptide antigen with preformed particles, have been shown to induce strong antigen-specific, cell-mediated and/or humoral immune responses to a wide range of antigens, including bacterial, viral, parasitic and cancer antigens [138,170]. There are ISCOMATRIX vaccines now approved for veterinary use and currently undergoing clinical trials for human use [171,172]. While it is known that the adjuvanting property of the particles is due at least in part to stimulating IL-12-dependent pathways, it seems to be independent of activating TLRs [170].

Exosomes, which are naturally occurring vesicles with a diameter of 50–90 nm, thought to be involved in cell-to-cell communication (including between immune cells) and in the selective removal of membrane proteins [173,174], have been exploited as another type of liposomal antigen carrier. Exosomes are secreted from many different types of cells and often contain MHC class I and II molecules, as well as other costimulatory molecules such as CD86 [175]. It has been demonstrated that delivering peptides to DCs through exosomes is far more effective than delivering free peptide [176]. One of the first studies to show that exosomes can present antigenic peptides and induce a protective immune response was performed by Aline and colleagues in which *Toxoplasma gondii*-pulsed, DC-derived exosomes stimulated antigen-specific humoral and cell-mediated responses against the pathogen [177]. However, there is evidence that exosomes require an additional adjuvant to effectively prime naive T_c cell responses *in vivo* [178]. Supporting this, the combination of exosomes and TLR3 (dsRNA) or TLR9 (CpG ODNs) agonists has been shown to successfully activate T_c cells, leading to tumor rejection in mice [179]. There are now a number of formulations developed for cancer immunotherapy that have entered into clinical trials [138,180].

Virus-like particles—Another design route for delivering peptide antigens is to incorporate them into noninfectious, nonreplicating virus-like particles (VLPs) [181]. VLPs are made from recombinant viral envelope and/or capsid proteins that self-assemble into 20–100-nm diameter structures capable of displaying multiple antigenic peptides on their surface. These peptides are derived either from the parental virus, from the genetic insertion or fusion of foreign antigenic epitopes, or by chemical conjugation of the peptides to pre-assembled VLPs. One of the few influenza vaccines available that uses adjuvants is made from a type of VLP called a virosome, marketed as Inflexal V® (Crucell, The Netherlands) [182]. Many other VLP formulations are currently in clinical or preclinical studies, and VLP-based hepatitis B virus and HPV vaccines are now licensed for human use [181].

Other pathogens have proven to be more challenging for VLP vaccines, such as those that directly infect immune cells, those that have developed mechanisms to evade the immune system and those that undergo rapid genetic drift. A number of studies show that the addition of TLR agonists as additional adjuvants can aid in the effectiveness of some VLPs. For example, TLR9-stimulating CpG ODNs packaged inside a VLP displaying a lymphocytic choriomeningitis virus T_c-cell epitope stimulated production of antigen-specific T_c cells and cured mice from the virus-induced tumor with a single dose of the VLP vaccine [183]. In another example, the TLR5 ligand flagellin was anchored into a H_1N_1 influenza VLP and compared with the same VLP construct but without the flagellin. The flagellin VLP induced higher levels of specific IgG antibodies in mice, but both VLPs provided full protection against viral challenge. Interestingly, however, the VLP with flagellin resulted in superior protection over the standard VLP upon challenge with the heterosubtype virus H_3N_2, demonstrating the

potential for cross-protective VLP vaccines [184]. Enhanced immunogenicity was also seen with another influenza virosomal vaccine upon incorporation of the nontoxic LPS analog LpxL1 [185].

Biodegradable polymers & solid-core beads—Synthetic particles made from degradable polymers such as polylactide-co-glycolides, poly D,L-lacticcoglycolic acid (PLGA), poly D,L-lactide and poly ortho esters have been widely explored as peptide antigen delivery vehicles [186]. They can serve as antigen reservoirs, slowly releasing peptides for days, weeks or even months, as dictated by the particle degradation rate, potentially eliminating the need for boosters. The diameter of these biocompatible particles can range from 10 nm to up to 250 μm, depending on the composition and method of preparation. It has been shown that microparticles undergo rapid phagocytosis by macrophages [187] and DCs [188], both *in vitro* and *in vivo* [189].

Various polymeric micro- and nanoparticles have demonstrated immune-stimulatory behavior, which can be enhanced when coformulated with both peptides and TLR agonists. For example, a single dose of a 300-nm PLGA nanoparticle loaded with a hepatitis B virus peptide antigen and MPLA induced a significantly stronger cell-mediated response with a predominant IFN-γ profile in mice than when using the free antigen, the free antigen with MPLA or particles loaded only with antigen [190]. Alternatively, TLR agonists can be adsorbed onto the particle surface. Recent work demonstrates, for example, the ability of poly(I:C)-coated 3-μm PLGA particles to prime DC maturation [191]. In another design, enhanced immunogenicity was seen when peptide antigen was adsorbed to the anionic surface of polylactide-co-glycolides microparticles with a mean size of 1 μm that contained either encapsulated MPLA or the synthetic LPS RC529 [192]. Recently, a new type of biodegradable particle has been described in which viral peptide epitopes are encapsulated in a SiO_2-templated hydrogel capsule and was shown to activate T_c cells *ex vivo* [193].

Other materials like gold, silica, latex and polystyrene have also been used to form solid-core nanobeads for peptide-based immunotherapy. Peptide-conjugated nanobeads can, without additional adjuvant, stimulate humoral and/or cell-mediated responses. For example, this was demonstrated in sheep when multiple peptides from the foot-and-mouth virus were either conjugated separately to individual 49 nm-polystyrene nanobeads or conjugated as a mixture of peptides on the beads [194]. It is possible that the adjuvant properties of peptide-conjugated nanobeads is simply due to their virus-like size, which allows them to interact with DCs [138]. Correlating nanobead size and immunogenicity, Fifis and colleagues showed that 40–50-nm polystyrene nanoparticles conjugated with tumor antigens and administered to mice colocalized with DCs in the lymph nodes provided both prophylactic and therapeutic effects [195].

Expert commentary

Vaccine development is a complex and daunting endeavor, yet at the same time there is enormous potential in peptide-based immunotherapy. By design, rationally engineered peptide antigen delivery systems can generate safer and more effective immunotherapy than traditional pathogen-based vaccines. Such delivery systems have the potential to minimize side effects by targeting specific APCs and linking antigen with adjuvant, allowing for greater quality control and easier validation. In addition, peptide-based vaccines can not only be used for prophylaxis, but also for therapeutic treatments. As evidence of the progress being made, many peptide-based formulations and TLR agonist adjuvants have entered clinical trials [67,68, 138,150,171,180,181], or have already been licensed for use [182,196].

The main challenge now facing the field is bringing more of these vaccines to the market. Peptide-based vaccines have been slow to reach commercialization, largely due to safety concerns regarding the accompanying adjuvants, and not necessarily because of a lack of efficacy. A comprehensive evaluation of all of the latest designs will be the next step to really advance peptide-based immunotherapy to the next level. Similarly, systematic experiments are needed that define which combined properties form the safest and most effective vaccines. To efficiently evaluate vaccine candidates, apply the lessons learned to new vaccine designs and to translate the results from the laboratory to the clinic and eventually to the market, a heightened level of communication between scientists and clinicians at all stages of vaccine development will be crucial.

Five-year view

A recurrent theme in recent peptide-based immunotherapy studies is to trigger adaptive, antigen-specific responses by first stimulating the innate immune system. Thus the next generation of vaccines will largely be designed to deliver defined antigenic peptides, target DCs (e.g., by size or receptor binding) and/or potentiate peptide immunogenicity via PRR agonists. Combining particulate carriers with TLR agonists will prove especially productive. New vaccines may also incorporate multiple TLR and other PRR agonists in order to take advantage of their synergistic effects on cytokine production in enhancing immune responses. Success of TLR-stimulating peptide vaccines in the next 5 years will correlate with progress in understanding TLR agonist adjuvanticity, and in finding a balance between effective immune stimulation and potentially excessive inflammatory responses.

Safety concerns will result in peptide-based vaccines first impacting diseases where the benefits of using such vaccines will be large, such as with cancer and HIV, making any potential risks associated with the adjuvants used more acceptable. This is already evident in the majority of ongoing vaccine clinical trials. As these vaccines come to market, they will provide new tools to control major global diseases that are difficult to control by other means. With a growing knowledge of the immunological mechanisms of adjuvant action, including identifying the roles of specific signaling pathways important in regulating adaptive immunity, and of antigen recognition, processing and presentation, peptide-based vaccines will become increasingly more tailored and therefore more effective therapeutics. The challenge will be in sculpting adaptive immunity to achieve the desired outcomes to immunotherapy in preventing and treating infectious and chronic diseases.

Key issues

- Peptide vaccine efficacy is determined by how the peptides are recognized and processed by the immune system. Specifically, peptide concentration, multivalency, secondary structure, length and the presence of helper T-cell epitopes can significantly affect the elicited immune response.
- Soluble peptides alone are weakly immunogenic and usually need to be coadministered with potentially toxic adjuvants. Adjuvants largely function by stimulating the innate immune system, particularly by activating dendritic cells (DCs).
- DCs are critical in linking the innate and adaptive arms of the immune system. Conserved microbial motifs can trigger innate responses, including through binding to Toll-like receptors (TLRs) on the surface of DCs and other antigen-presenting cells (APCs). Multiple TLR ligands can act synergistically to upregulate cytokine production by DCs, initiating different types of immune responses.

- As TLR agonists can elicit strong inflammatory responses, one of the major hurdles for their use in vaccines will be to deliver them in a way to ensure acceptable safety profiles.
- Linking peptide antigens with TLR agonists in a single construct has proven to be an effective approach to enhancing peptide immunogenicity.
- Many sophisticated designs for delivering antigenic peptides and adjuvants have been explored, including direct peptide–adjuvant conjugates, and particulate systems such as liposomes, virus-like particles, degradable polymers and nondegradable solid-core beads.
- These delivery vehicles can not only couple peptide antigens with TLR agonists, but can have immune-stimulating properties, such as DC targeting, multivalent peptide display and additional adjuvant activity, and can provide protection against degradation.
- Peptide-based vaccines incorporated into nano-sized particulate carriers have demonstrated a considerable potential as new and improved prophylactic and remedial immunotherapeutics.

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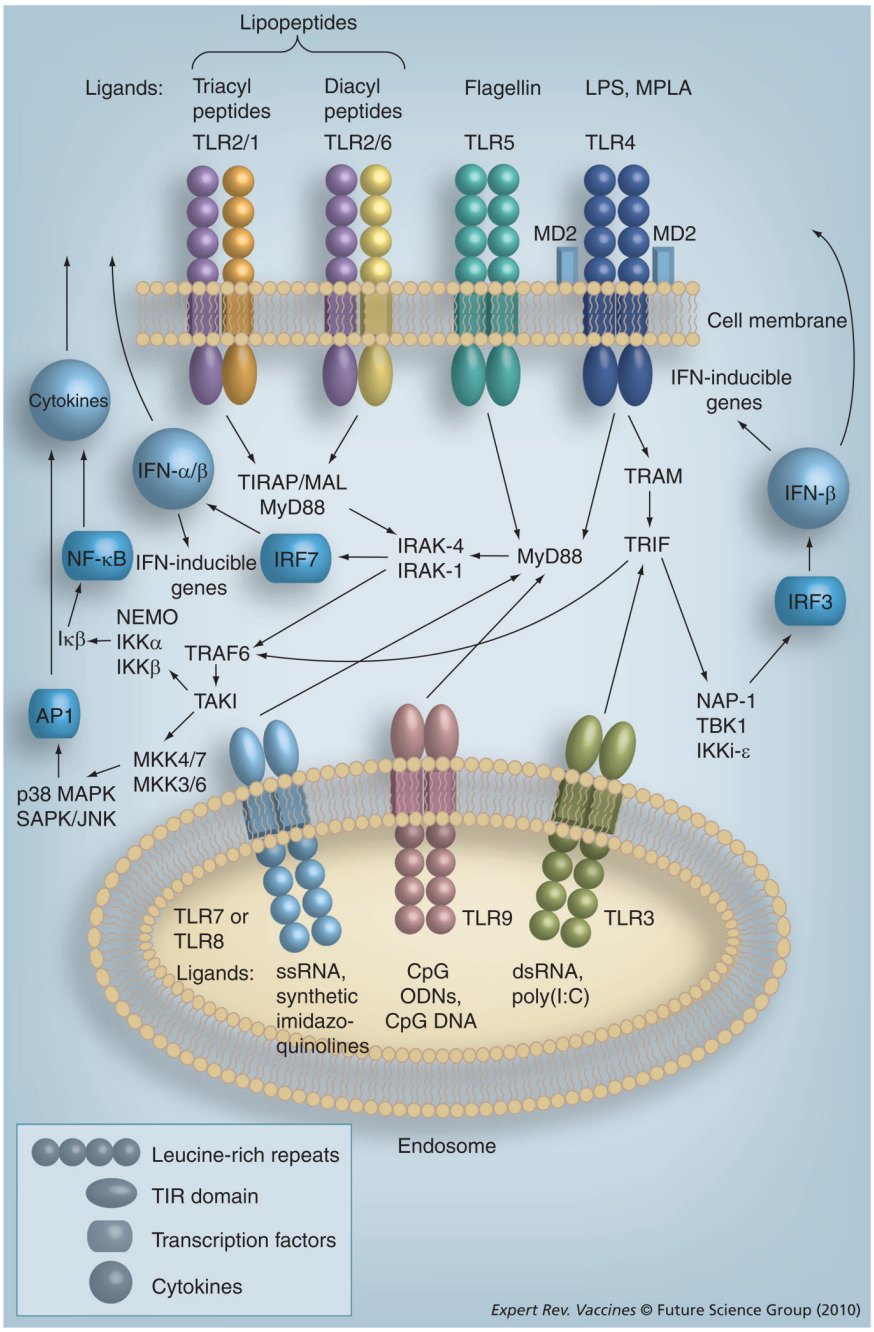

Figure 1. Toll-like receptor signaling pathways

The TLR proteins initiate complex intracellular signaling pathways, resulting in the production of various cytokines that dictate specific immune responses. Typical ligands for the TLR domains are shown. See text for relevant references.

AP1: Activator protein 1; IFN: Interferon; IKK: IκBα kinase complex; IRAK: IL-1 receptor-associated kinase; IRF: Interferon regulatory factor; MAL/TIRAP: MyD88 adapter-like TIR domain-containing adapter protein; MAPK: Mitogen-activated protein kinase; MKK: Mitogen-activated protein kinase kinase; MyD88: Myeloid differentiation primary-response gene 88; NAP: NF-κB-activating kinase-associated protein; NEMO: NF-κB essential modulator; ODN: Oligodinucleotide; SAPK/JNK: stress-activated protein kinase/c-Jun NH_2-

terminal kinase; TAK: Transforming growth factor-β-activated kinase; TBK: TANK-binding kinase; TIR: Toll/IL-1 receptor; TLR: Toll-like receptor; TRAF: TNF receptor-associated factor; TRAM: TRIF-related adapter molecule; TRIF: TIR-domain-containing adapter inducing IFN-β.

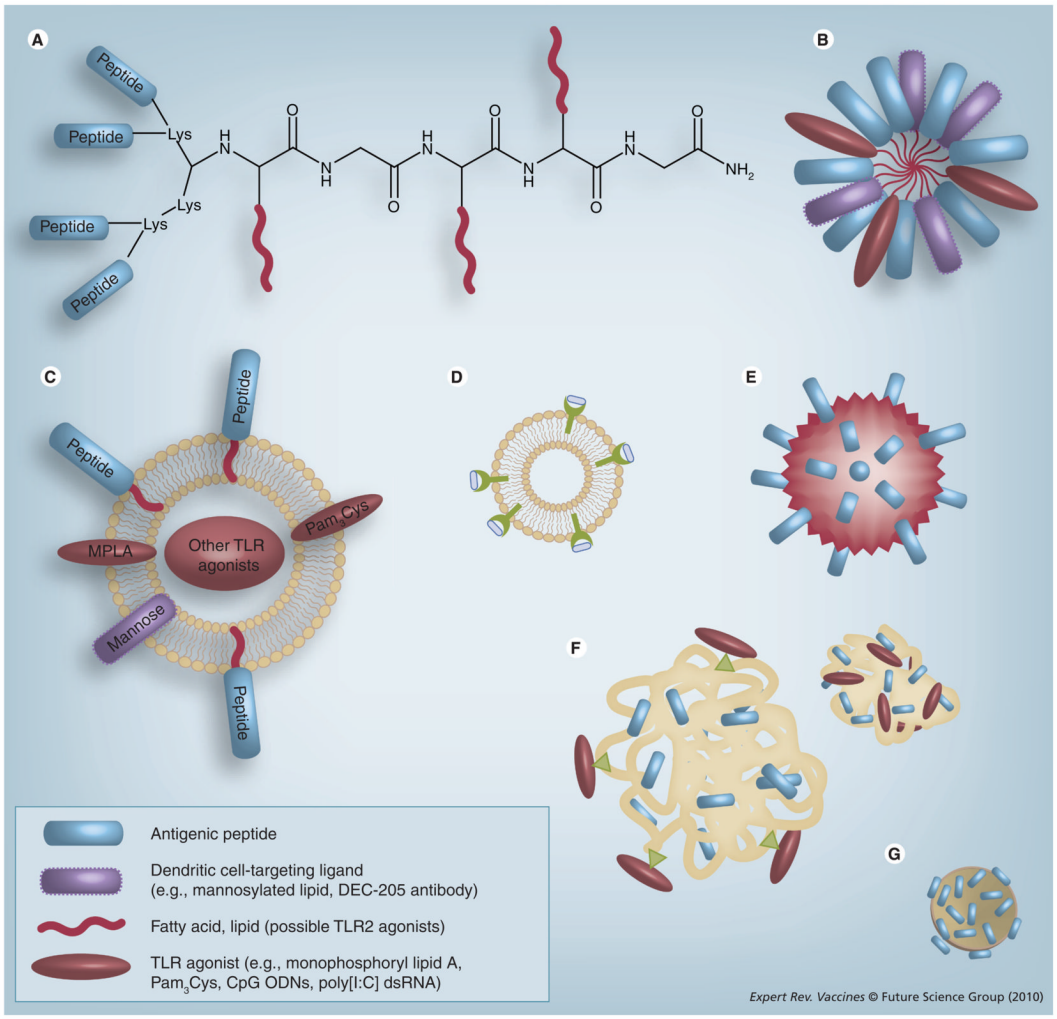

Figure 2. Possible peptide antigen delivery systems

Peptide antigen–adjuvant conjugates such as **(A)** lipid–core peptides; **(B)** peptide amphiphiles self-assembled into mixed micelles (protein analogous micelles; lipid-based carriers including **(C)** synthetic, multifunctional vesicles and **(D)** endogenous exosomes containing peptide-loaded MHC molecules; **(E)** noninfectious virus-like particles displaying recombinant peptide antigens; **(F)** microparticles and nanoparticles made from many types of polymers with encapsulated or surface-conjugated peptides; and **(G)** solid-core nanobeads with conjugated peptides.

MPLA: Monophosphoryl lipid A; ODN: Oligodinucleotide; Pam_3Cys: Tripalmitoyl-*S*-glyceryl cysteine; TLR: Toll-like receptor.

Table 1

Natural ligands and pathogens that bind Toll-like receptors and examples of vaccine adjuvant candidates for Toll-like receptors.

TLR	Natural ligands	Pathogens	Vaccine adjuvant candidates
TLR1/2	Triacylated lipopeptides	Bacteria	Pam_3Cys
TLR2/6	Diacylated lipopeptides Peptidoglycan Zymosan LTA	*Mycoplasma* bacteria Bacteria Fungi Gram-positive bacteria	Pam_2Cys, MALP-2, other hydrophobic groups?
TLR3	dsRNA	Viruses	Poly(I:C)
TLR4	LPS	Gram-negative bacteria	MPLA, other LPS analogues
TLR5	Flagellin	Bacteria	Flagellin
TLR7	ssRNA	Viruses	ssRNA, imiquimod, resiquimod, adenosine and guanosine derivatives
TLR8	ssRNA	Viruses	ssRNA, adenosine and guanosine derivatives, resiquimod
TLR9	CpG DNA	Bacteria, viruses, protozoa	Unmethylated CpG ODNs
TLR10	Unknown		None discovered

CpG DNA: Cytosine-guanine rich DNA; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid;

MALP-2: Macrophage-activating lipoprotein-2; MPLA: Monophosphoryl lipid A; ODN: Oligodinucleotide;

Pam_2Cys: Dipalmitoyl-*S*-glyceryl cysteine; Pam_3Cys: Tripalmitoyl-*S*-glyceryl cysteine;

poly(I:C): Polyriboinosinic-polyribocytidylic acid; TLR: Toll-like receptor.