More than one reason to rethink the use of peptides in vaccine design

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Abstract | The use of peptides as therapeutics is experiencing renewed enthusiasm owing to advances in delivery, stability and design. Moreover, there is a growing emphasis on the use of peptides in vaccine design as insights into tissue-specific processing of the immunogenic epitopes of proteins and the discovery of unusually long cytotoxic T-lymphocyte epitopes broaden the range of targets and give clues to enhancing peptide immunogenicity. Peptides can also be synthesized with known post-translational modifications and/or deliberately introduced protease-resistant peptide bonds to regulate their processing independent of tissue-specific proteolysis and to stabilize these compounds *in vivo*. We discuss the potential of peptide-based vaccines for the treatment of chronic viral diseases and cancer, and review recent developments in the field of peptide-based vaccines.

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Vaccination is one of the most successful public health initiatives ever achieved, with the global eradication of diseases such as smallpox and the virtual eradication of poliomyelitis. Despite such noteworthy successes, vaccines for many diseases remain elusive and as such several strategies have been devised to deliver specific and immunogenic vaccine components to the immune system in the hope of eliciting a therapeutic or prophylactic immune response. Traditionally, vaccines have consisted of live attenuated microorganisms or inactivated microorganisms delivered by injection. However, many pathogenic microorganisms are difficult to culture *in vitro*, and therefore production of live attenuated or inactivated vaccines of these pathogens are impractical. Moreover, features of even attenuated microorganisms may result in detrimental immune responses, or the pathogen may contain material that initiates unwanted host responses. As our knowledge of the targets of immune responses has grown, so too has the sophistication with which vaccines are designed and delivered. Thus, it is often a beneficial strategy to select particular protein antigens from the microorganism for inclusion into recombinant vaccines. Focusing on a limited set of antigens is also relevant to cancer vaccination in which tumour cells or lysates may contain predominantly normal self-proteins that are of no therapeutic benefit, or contain material that potentially carries the capacity to induce further malignancy. Therefore, the identification and administration of tumour-specific antigens rather than crude tumour preparations is also highly desirable in this case.

Taking this reductionist approach one step further, the most precise selection of vaccine components exists in epitope-based peptide vaccines, which are the subject of this Review. These peptide epitopes represent the minimal immunogenic region of a protein antigen and allow for precise direction of immune responses. As yet, there are no human peptide-based vaccines on the market — this stems primarily from the difficulties associated with peptide stability and delivery, and the challenge posed by the diversity of human immunogenetics. However, similar to therapeutic peptides that have been investigated for other indications, strategies have become available to modify peptides to enhance both their immunogenicity and stability, which will be discussed below. Given the long development time experienced with therapeutic peptides, and their recent emergence into the pharmaceutical arena, it is likely that peptide-based vaccines will enter the human therapeutics marketplace in the near future. Several such vaccines are currently in development (TABLE 1a,b).

In this Review, after briefly introducing the key immunological concepts of vaccine design, we will discuss the latest developments in the design of peptide vaccines that have led to the return of epitope-based strategies to the forefront of vaccine design.

Key concepts of peptide vaccine immunology

The adaptive immune system comprises two arms, one that is responsible for the cytotoxic immune response and one for the humoral immune response. Cytotoxic T lymphocytes (CTLs; also referred to as $CD8^+$ T cells) eradicate

infected cells or tumour cells through direct cytotoxic action on these targets. B cells are the main players of the humoral immune response and generate antibodies towards pathogen-derived molecules. Both processes are further dependent on T-helper cells (T_H cells; also referred to as $CD4^+$ T cells). CTLs detect infected or malignant cells through the recognition of major histocompatibility complex class I (MHC class I) molecules that are complexed with peptides derived from proteins expressed within the cell. T_H cells recognize MHC class II molecules that are complexed to peptides derived from predominantly exogenous proteins. All nucleated cells present peptides that are derived from intracellular proteins on their surface bound to MHC I, whereas peptides derived from extracellular proteins are mainly presented by MHC II on specialized antigen-presenting cells (APCs), such as dendritic cells and macrophages. In both cases the T-cell receptor (TCR) on the surface of the CTL or T_H-cell forms a complex with the MHC I/peptide-epitope complex or the MHC II/peptide-epitope complex, respectively; these interactions are aided by the CD8/CD4 co-receptors, respectively. The intricate interplay of these peptide-dependent recognition processes results in the propagation of immune responses that control infection and cancer in humans as depicted in FIG. 1. The goal of vaccination is to induce immunity towards these states by selectively stimulating antigen-specific CTLs or B cells and T_H cells. A vaccine must therefore contain two antigenic epitopes: a T_H-epitope and an epitope that will either induce specific B-cell or CTL responses. In some cases these can

Table 1a | **A selection of peptide-based vaccines at various stages of development**

Company (url)	Vaccine	Notes
Launched peptide-based vaccines		
Pfizer (http://www.pfizer.com)	Immunocastration of pigs and companion animals, Improvac	Gonadotropin-releasing hormone (GnRH)-based vaccine to remove boar taint
Phase III		
Sanofi Pasteur (http://www.sanofipasteur.com)	Gastric cancer vaccine	Based on gastrin-related peptides
Phase II/III		
Avant Immunotherapeutics (http://www.avantimmune.com)	Hypolipidaemic, CETi-1 vaccine	Targets cholesteryl ester transfer protein
Bionor Immuno (http://www.bionorimmuno.com)	AIDS vaccine, Vacc-4X	HIV p24 peptide
Phase II		
Novartis (http://www.novartis.com)	Allergy Vaccine	Anti-asthmatic
Immune Response Corporation (http://www.imnr.com)	NeuroVax	Multiple sclerosis (MS) treatment consisting of three T-cell receptor (TCR) peptides (BV5S2, BV6S5 and BV13S1) that represent immunogenic regions from TCRs expressed by pathogenic T cells in over 90% of patients with MS
Intercell (http://www.intercell.com)	IC-41	Targets hepatitis C virus
Alteris Therapeutics (http://www.alteristhera.com)	ALT-110	Peptide based on the epidermal growth factor receptor type III deletion mutant (EGFRvIII) cancer antigen that is currently being studied in a Phase II clinical trial for brain cancer and a Phase I clinical trial for various other cancers
Neurocrine Biosciences (http://www.neurocrine.com)	NBI-6024	Altered peptide ligand based on insulin for treatment of type I diabetes
Memorial Sloan–Kettering Cancer Center (http://www.mskcc.org)	CMLVAX100	Targets leukaemia by using the BCR–ABL junction peptide
Pharmexa (http://www.pharmexa.com)	GemVax	Vaccine for treatment of colorectal and gastric cancers with the microsatellite instability (MSI) phenotype. The vaccine consists of peptides reflecting frameshift-mutated transforming growth factor-β receptor 2 (TGFβR2) and BCL-2-associated X protein (BAX) protein sequences frequently found in MSI tumours
Pharmexa	GV1001	Vaccine based on a combination of telomerase vaccine and p21 Ras peptides for treatment of pancreatic, colorectal and lung cancer
Sanofi Pasteur	Canarypox-based vaccine (ALVAC) plus peptide boost	ALVAC AIDS vaccine plus peptides, ALVAC-MAGE1,3 plus peptides, and ALVAC-gp100 plus peptides for skin cancer

Major histocompatibility complex
(MHC). A cluster of genes on human chromosome 6 or on mouse chromosome 17 that encode for MHC molecules. These molecules are the most polymorphic in the genome, and are the ones recognized by T lymphocytes during transplant rejection. They encode for a family of cellular antigens that help the immune system to recognize self from non-self.

Dendritic cells
Antigen-presenting cells found in T-cell areas of lymphoid tissues, but also as minor cellular components in most tissues. They have a branched or dendritic morphology and are the most potent stimulators of T-cell responses.

Macrophages
One of the main types of professional phagocytes. Macrophages are long-lived and detrimental for many microbial pathogens. Intracellular bacteria, including *Mycobacterium tuberculosis*, can survive within the macrophages.

overlap substantially within the sequence of an antigen, and in others they might be present in discrete regions of the antigen or present in different antigens from the targeted pathogen.

Synthetic peptides as vaccines

Compared with conventional vaccines, which are based on attenuated or inactivated microorganisms, synthetic peptides offer several advantages over other forms of

Table 1b | **A selection of peptide-based vaccines at various stages of development**

Company (url)	Vaccine	Notes
Phase I		
Antigenics (http://www.antigenics.com)	AG-707	Vaccine against genital herpes caused by herpes simplex virus-2 (HSV-2). Based on AG-702, an earlier vaccine that contained a single antigen. It consists of a recombinant human heat-shock protein (HSP) complexed with 32 synthetic peptides from HSV-2 proteins
Crucell and Pevion (http://www.crucell.com; http://www.pevion.com)	Malaria vaccines	Based on two synthetic peptides, which mimic the native structure of important antigens of the malaria parasite.
GSK (http://www.gsk.com)	Breast cancer vaccine, prostate cancer vaccine	HER-2/neu (also known as ERBB2)-peptide microspheres that target breast cancer and microspheres containing proprietary prostate-antigen-derived peptides
DOR Biopharma (http://www.dorbiopharma.com)	RiVax	Based on ricin-toxin-derived peptides
Millenium Biologix (http://www.millenium-biologix.com)	*Pseudomonas* vaccine, Cytovaxine	Consensus sequence peptide vaccine used to prevent *Pseudomonas aeruginosa* infection, which is among the leading causes of hospital-acquired pneumonia
Wyeth (http://www.wyeth.com)	HIV-CTL multi-epitope peptide vaccine formulated with RC529-5E and GM-CSF	Synthetic peptide, multi-epitope vaccine for AIDS
National Cancer Institute (Rosenberg laboratory) (http://www.cancer.gov)	Designer peptide vaccine	Designer peptide vaccine based on gp100 for treatment of melanoma
Generex (http://www.generex.com)	Invariant-chain peptide fusions	Vaccines targeting infectious diseases (HIV, H5 influenza, *Vaccinia*) and cancer (melanoma, HER-2/Neu)
Ludwig Institute for Cancer Research (http://www.licr.org)	Peptide-based vaccines for an array of cancers	Based on testes cancer class of antigens and epitopes mapped from them. For example, NY-ESO-1 and MAGE
Preclinical development		
Crucell and Bachem (http://www.bachem.com)	Various vaccines	For treating Alzheimer's disease, breast cancer, melanoma
MDM Group Inc. (http://www.mdmgroupinc.com/biodefense.php)	Smallpox vaccine	Vaccine is based on the Maksyutov Antigenic Peptide technology platform
DOR Biopharma	BT-VACC	Targets botulinum toxin
Immunotope (http://www.immunotope.com)	Ovarian and colon cancer related peptide cocktails that target heterogeneously expressed antigens	For treating colon cancer, ovarian cancer
Mymetics and Pevion (http://www.mymetics.com)	HIV vaccine	HIV gp41-derived peptides fused to a virosome
Neovacs (http://neovacs.com)	E7 toxoid	For treating cervix cancer
Pepscan systems (http://www.pepscan.nl)	Prostate cancer vaccine	Anti-gonadotropin-releasing hormone (GnRH)
Pevion	Breast cancer vaccine	Based on HER-2/Neu peptides fused onto a virosome
Prana Biotech (http://www.pranabio.com)	Vaccine for Alzheimer's disease, PBT-2	Targets β-amyloid
Vaccine Solutions (http://www.www.vaccine-solutions.com.au)	Vaccines targeting infection diseases	Targets cytomegalovirus, *Streptococcus*

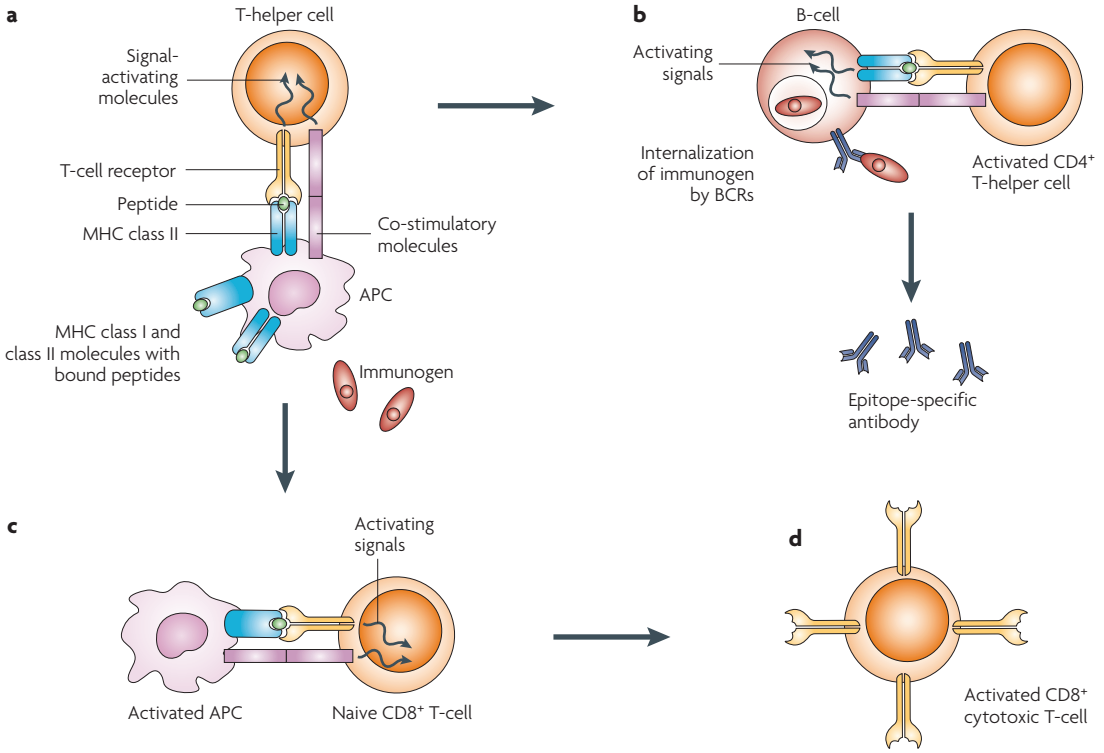


Figure 1 | **The interplay of antigen-presenting cells, T and B cells in the immune response.** The first step in the generation of an antibody response is the uptake of an immunogen by an antigen-presenting cell (APC). Antigens undergo proteolysis to form peptides, some of which are bound by major histocompatibility complex (MHC) class II molecules that are then transported to the APC surface. T-helper cells (T_H cells; also known as $CD4^+$ T cells) that bear receptors capable of interacting with the peptide/MHC II complexes can then interact with the APC (**a**). Additional interactions occur through co-stimulatory molecules that are expressed on APCs and their ligands on the T_H-cell. These recognition events result in the transmission of activation signals to the T_H-cell, and the activated T_H-cell is now poised to respond to those B cells that display the same peptide/MHC II complexes on their surfaces, acquired as a result of the internalization of the immunogen through specific surface-immunoglobulin receptors (for example, B-cell antigen receptors (BCRs) (**b**). It is this interaction between T_H cells and B cells that is termed 'help,' and results in the triggering of the B-cell to differentiate into a plasma cell that is capable of secreting antibody of the same specificity as that of the immunoglobulin receptor. The interaction of activated T_H cells with certain subsets of APC can also bring these to a state capable of stimulating naive $CD8^+$ T cells (**c**). Presentation of appropriate peptide epitopes to a naive $CD8^+$ T cell by an activated APC results in the generation of cytotoxic T cells (CTLs) that are able to recognize and kill target cells that display a viral or tumour peptide in the context of MHC class I molecules (**d**). Cytokines are also produced by each cell type, which profoundly influence the type of immune response that is elicited.

vaccines, particularly with regards to safety and ease of production (BOX 1). However, drawbacks include poor immunogenicity of the simple peptides and the need to potently stimulate T cells and elicit immunological memory. Adjuvant science, lipopeptide conjugation and direct delivery to dendritic cells are some of the approaches currently being used to overcome these problems (see BOX 2 and below).

Use of epitope-based vaccines are also restricted to patients of a given tissue type (human leukocyte antigen (HLA) haplotype), and as such need to be tailored to accommodate the natural variation in *HLA* genes. Although initially thought to be a major impediment, new technologies have made this personalized-medicine approach feasible[1]. Moreover, the existence of HLA supertypes can simplify the use of epitope-based vaccines that target MHC-I-restricted cytotoxic T cells to essentially nine superfamilies with shared peptide-binding specificity and shared epitope presentation (Supplementary information S1 (table)). Supertype motifs generally allow for a significant reduction in the number of epitopes required to give excellent population coverage for a given pathogen; however, it should be noted that supertypes are not always predictive of stable peptide binding and significant variations, even between closely related alleles, can occur[2–4]. The binding of class II MHC ligands is more promiscuous and as such motifs have been difficult to delineate with any reliability. An alternative approach to the delivery of peptides with a broad relevance to different HLA allotypes is to use longer peptides and to rely on their accurate processing to shorter allele-specific peptides. The use of longer peptide precursors is not always desirable as tissue-specific processing and the frequent non-concordance of processing of longer exogenous peptides compared with endogenous antigens can result in unwanted immune responses (disussed in more detail later).

Human leukocyte antigen (HLA). Genetic designation for the human major histocompatibility complex.

Box 1 | **Advantages of peptide-based vaccines**

- The absence of infectious material, which can compromise many live or attenuated vaccines. Furthermore, many pathogens can be difficult or impossible to culture by conventional methods.
- The ability to exclude deleterious sequences from full-length antigens or other pathogen-derived molecules. Such sequences may be oncogenic, for example the Epstein–Barr virus nuclear antigens (EBNA) of the Epstein–Barr virus[78], or implicated in autoimmune diseases, for example the M protein of group A *Streptococci*[79,80].
- Lipid, carbohydrate and phosphate groups can be readily introduced in a controlled manner to improve immunogenicity, stability and solubility.
- Peptides are easily characterized and analysed for purity using well-established analytical techniques such as liquid chromatography and mass spectrometry. This facilitates quality control and ultimately approval by the regulatory authorities.
- The production of chemically defined peptides can be carried out economically on a large scale.
- Peptide preparations can be stored freeze-dried, which avoids the need to maintain a 'cold-chain' facility in storage, transport and distribution.
- There is no risk of reversion or formation of adverse reassortants that can lead to virulence, which is a potential problem with live attenuated vaccine preparations.
- There is no risk of genetic integration or recombination, which is a problem facing regulatory authorities that are dealing with DNA vaccines.
- Peptide-based vaccines can be designed to include multiple determinants from several pathogens, or multiple epitopes from the same pathogen.
- The introduction of non-natural amino acids and peptide-like molecules into peptide-based vaccines allows the design of more drug-like compounds, which opens up avenues for vaccine delivery and rational drug design in vaccinology.

The administration of a peptide epitope taken out of context of the whole antigen can be challenging as the exogenously administered peptide will not necessarily follow the same pathway of processing as the native pathogen or the cancer-cell derived antigen, and consequently might not elicit the same type of immune responses (BOX 3). Furthermore, in order to activate B cells to generate a specific antibody response to a given antigen, the peptide epitope needs to possess a conformation that is similar to that of the native antigen. Several approaches are available to induce peptides to fold correctly[5], but any approach in which conformational elements are incorporated requires some knowledge of the structure of the native antigen. The induction of CTL responses on the other hand has little requirement for epitope conformation, and simple peptide epitopes composed of 8–10 amino acids are able to induce cytotoxic responses. As the induction of CTL and B-cell responses are generally dependent on the additional stimulation of T_H cells, a typical approach to peptide-epitope vaccine design is to covalently couple the antibody or CTL epitope to a carrier protein, such as tetanus toxoid, which provides a source of T_H epitopes. In a clinical setting, the provision of T-cell help is more readily achieved using defined T_H epitopes that are matched to the haplotype of the patient[5].

Peptide binding to MHC I. CTLs are generally activated by antigens that are derived from intracellular proteins, which are presented in a complex with MHC I on the surface of cells. Intracellular proteins (for example, viral proteins) are degraded to oligopeptides in the cytoplasm through the action of a multicatalytic protease structure known as the proteasome. These peptides are transported into the lumen of the endoplasmic reticulum where they assemble with MHC I and transit through the Golgi to the cell surface (BOX 3). The path that exogenously administered extracellular peptides follow post-immunization is poorly understood, although it appears that the extracellular environment is rich in proteases and peptides are rapidly cleared from the body. Peptide-based vaccination strategies therefore must take into account the inherent instability of native peptides. Importantly, to design effective peptide-epitope-based vaccines, the requirements for their incorporation into the MHC I/peptide complex need to be better understood.

MHC I molecules consist of a polymorphic heavy chain, a monomorphic light chain (β2 microglobulin) and an antigenic peptide ligand (corresponding to the antigenic epitope of a given protein). The heavy chain forms an antigen-binding groove that can accommodate antigenic peptides that are typically 8–10 amino-acid residues in length (Supplementary information S2 (figure)). Amino-acid residues that line the binding groove are the focus for most MHC polymorphisms that determine the peptide-binding specificity of allelic forms of MHC molecules. Structural and biochemical studies of bound peptides have contributed significantly to our knowledge of the binding characteristics of different HLA (MHC I) alleles (Supplementary information S1 (table)). In particular, these studies have highlighted the role of conserved amino acids at different positions of all allele-specific peptide ligands that are involved in binding to the cleft of MHC I molecules (anchor residues). The studies have also highlighted the role of the more variable amino acids at other positions within the peptide that project out of the antigen-binding cleft and make crucial specificity-determining contacts with receptors on the surface of T cells. This approach has allowed the identification of specific binding motifs, which have been used to successfully predict T-cell epitopes. Listings of motifs are conveniently web based (for example, SYFPEITHI, a database of MHC ligands and peptide motifs). However, the success rate for *de novo* prediction of T-cell epitopes, even for well-studied and abundant MHC alleles, is only about 60% for many alleles (and for new alleles or MHC I molecules from poorly studied ethnic populations no binding motifs are available). However, recent studies have improved the predictive capacity of algorithms for some well-studied alleles substantially[3,6–10]. Reasons for poor predictability include the occurrence of non-motif-based ligands, peptides of unusual length, post-translationally modified ligands or the failure of antigen processing to liberate the predicted peptides[7,11–17]. Furthermore, many T-cell responses are focused on one or two immunodominant peptides that are selected from the numerous potential MHC ligands of a given pathogen[18]. The participation of so few epitopes limits the number of distinct epitopes that are required in a peptide-based vaccine to elicit a protective immune response. Thus, predictive markers of immunogenicity must take into account not only peptide binding but also the abundance and density of the antigen that is

Box 2 | **Developments in human adjuvants and peptide vaccine delivery**

Use of biological macromolecules as adjuvants. The covalent or non-covalent attachment of peptides to proteins that generally have some role in the innate immune response or target the proteins to receptors on antigen-presenting cells (APCs) has been investigated as a mode of delivery, and as an adjuvant for bound peptides. Conjugates of peptide antigens with heat-shock proteins[81–88] and ligands of toll-like receptors (TLRs)[89] are examples of this approach.

Lipopeptides that target TLRs. The use of lipopeptides has been extensively characterized as a way of making peptide-based vaccines self-adjuvanting[90]. These peptides appear to operate by targeting the vaccine to APCs that express TLRs and are internalized. They also signal through these receptors to induce dendritic cell (DC) maturation[90].

Recombinant cytokines. Recombinant cytokines can be used as an adjuvant in peptide formulations[89]. These include molecules such as granulocyte-macrophage colony-stimulating factor (GM-CSF)[91,92] and interleukin 12 (IL12)[93,94], which have been used in human clinical trials.

Oil-emulsion-type adjuvants. In general, oil-emulsion-type adjuvants that are traditionally used in vaccine studies in experimentally tractable rodents are too toxic for human prophylactic vaccine use, although they might be suitable for use in terminal conditions such as cancer. Some oil-based adjuvants have been approved for human use such as Montanide[95].

Particulate delivery systems. In addition to having a depot effect on the peptide antigens, inherent properties of the particles themselves engender immunogenicity of the peptides, and allow uptake of an immunogenic package of peptides and other molecules[96]. Included in this category are ISCOMs (immunostimulatory complexes)[97,98], liposomes[99], exosomes[100] and virosomes[101].

Polysaccharide adjuvants. Polysaccharide adjuvants that are based on inulin, a plant-derived oligosaccharide, can induce cellular and humoral immunity, and have good safety and tolerability profiles in humans[102].

Alum. Although able to induce a good antibody response and is widely accepted as a safe adjuvant in humans, alum has little capacity to stimulate cellular immune responses.

Cell-based delivery systems. The use of cultured DCs are particularly apt for the delivery of peptide vaccines. The direct surface loading of *in vitro* activated autologous DCs bypasses the processing requirements and allows for precise delivery of peptide antigens to the immune system. Extensive literature on the use of DCs as a delivery mechanism of peptides as vaccines exists[74,88,89].

present on the cell surface; the time of expression of the antigen during the infection or pathological process; the correct processing and luminal transport of the epitope; and the available T-cell repertoire in the host organism. Nonetheless, epitope prediction remains a popular first-screening method to identify candidate T-cell determinants for subsequent biological validation, and predictive algorithms are frequently combined with *in vitro* MHC-binding assays to confirm that the predicted ligands bind to the targeted MHC molecule[19,20].

Peptide binding to MHC II. The humoral immune response (antibody production by B cells) is generally induced in response to extracellular antigens, which are presented in a complex with MHC II on the surface of APCs (for example, dendritic cells, macrophages and B cells). In cells that are capable of receptor-mediated uptake of immune complexes (for example, Fc-receptor-positive cells), exogenous antigen is endocytosed via receptor-mediated processes and degraded in the early and late endosomes. These compartments are also the destination of antigens that are taken up less selectively by highly phagocytic APC-like dendritic cells and macrophages. The late endosomes therefore provide a rich reservoir of antigenic peptides that are transported to the cell surface for display and scrutiny by T_H cells. This process is mechanistically and physically distinct from the MHC-class-I-processing pathway (FIG. 2). Likewise, MHC II is physically distinct from MHC I and is composed of two polymorphic polypeptide chains (α and β) that form an $\alpha\beta$ heterodimer, which forms a binding cleft that accommodates peptide antigen. B cells that bind to a given antigen via their clonally distributed surface-immunoglobulin receptor, and display a specific MHC II/peptide complex by virtue of the capture of antigen, can be triggered to differentiate into antibody-producing plasma cells with the help of T_H cells (FIG. 1).

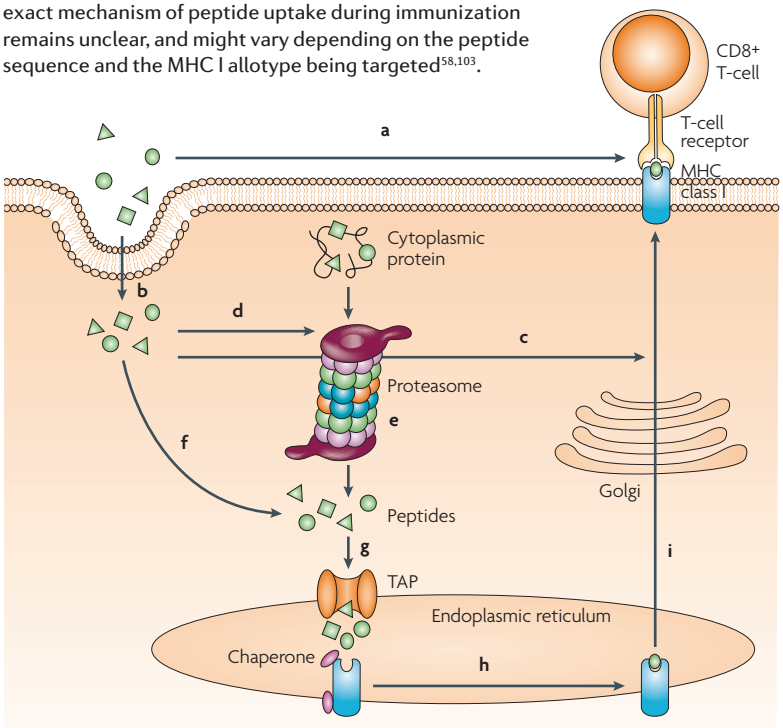
The mode of binding and repertoire of peptide ligands that are bound by MHC II differs from the binding of peptides to class I molecules. The interactions that close the peptide-binding cleft of class I molecules are not apparent in MHC II, which allows the termini of the bound class II peptide to project out of the ends of the cleft. Hence, MHC II peptides are typically longer than class I ligands, averaging around 13 amino-acids in length, but can be considerably longer. Similar to MHC I, polymorphic amino-acid residues line the pockets of the binding cleft. Both structural and biochemical studies indicate that amino-acid side chains at residues 1, 4, 6 and 9 of the class-II-bound peptide typically interact with these pockets, therby conferring allelic specificity[21] and 'anchoring' the peptide into the cleft. It has also been suggested that the binding of ligands to MHC II is more promiscuous than the binding of peptides to MHC I, owing to their free termini and ability to use a range of anchor residues or induce a shift in the binding register. This observation makes it more difficult to define anchor residues and to predict which peptides will be able to bind to particular MHC II molecules. Amino- and carboxy-terminal exopeptidase activities can trim peptides that are bound to class II molecules during their transit to the cell surface, further hampering efforts to define MHC-II-binding motifs and to reliably predict MHC II ligands[22].

Novel insights into the nature of T-cell epitopes

CTL epitopes of unusual length, and conformational dependence of their recognition. Despite antigen processing in the class I compartment predisposing towards peptides of 8–10 amino acids in length, peptides considerably longer than this bind to and are naturally presented by MHC I. These include naturally presented self-peptides of which at least 5% are over 10 amino acids in length[23]. The structures of several peptides that are considerably longer than 10 residues in complex with MHC I have been studied, and, in each case, the peptide adopts a bulging conformation while maintaining a conserved hydrogen-bonding network at the peptide amino termini and carboxy termini[24–28]. These bulged epitopes can be rigid and display a defined conformation or exhibit a considerable degree of flexibility when bound to the antigen-binding cleft. For example, we have recently studied a highly immunogenic region from the BZLF1

Box 3 | **Entry of peptide vaccines into the MHC I pathway**

The classical major histocompatibility complex (MHC) class I pathway involves the processing of antigen that is synthesized in the cytoplasm or has made its way into the cytoplasm (see figure below). The introduction of vaccine components into the class I pathway, therefore, is different to the normal processing of endogenous antigen and requires the peptides that are included in the vaccine to be exposed to a different environment than that of their cellular or pathogen-encoded counterparts. Despite being a common practice in experimental systems and in human clinical trials the exact mechanism of peptide uptake during immunization remains unclear, and might vary depending on the peptide sequence and the MHC I allotype being targeted[58,103].



High affinity peptides, or peptides that are capable of binding to alleles that express a high proportion of peptide-receptive molecules at their cell surface (that is, molecules with a cohort of low affinity endogenous ligands, for example, human leukocyte antigen-B27 (HLA-B27), B*4405), may load onto the MHC class I complex directly at the cell surface of antigen-presenting cells (APCs)[52,57,103] (**a**). The loading of exogenously administered peptides to MHC I is likely to mirror cross-presentation[104], a naturally occurring process whereby exogenous antigen is delivered into the APC for subsequent processing by the classical MHC I pathway. The extracellular administration of the peptide vaccine results in the exposure of the peptide to an array of extracellular proteases and cell-surface proteases that may result in differential trimming or destruction of the epitope compared with the processing of their cellular or pathogen-encoded counterpart. Cellular uptake of many peptides may be a prerequisite for MHC I loading. It is unclear how this process occurs but most probably results from macropinocytosis of peptides from the extracellular milieu by the highly phagocytic APC[54,105–107] (**b**). A role for heat-shock proteins (HSPs) in chaperoning these peptides or assisting in this process has been demonstrated in several studies[88,108,109]. At this point peptides may intersect the secretory pathway through fusion of endosomal compartments (**c**), or may escape from the endosomal/lysosomal system and enter the cytosol (**d**), where they are transported to the endoplasmic reticulum (ER). This may involve additional processing by the proteasome (multiple forms of the proteasome exist and can result in tissue or cell-specific processing of antigen and peptide-vaccine components) (**e**), or direct trafficking to the ER. Peptides are then actively transported into the ER by the transporter associated with antigen processing (TAP) (**f**). Cytosolic and ER-resident peptidases may further trim (or destroy) peptide epitopes during these processes (**g**). Translocated peptides are rapidly loaded into the antigen-binding cleft of class I molecules aided by several chaperones[110] (**h**). An alternative source of peptides, and perhaps the majority of endogenous MHC I ligands, come from defective ribosomal initiation products[111,112]. Once MHC I molecules are loaded with peptide, the epitopes are protected from further proteolysis and the complexes traverse the Golgi apparatus on their way to the cell surface where they are scrutinized by cytotoxic T lymphocytes (also referred to as $CD8^+$ T cells) (**i**).

antigen of the Epstein–Barr virus (EBV) that is presented by the HLA-B35 family members in a length and conformation-specific manner[15]. Some of these unusually long epitopes contain within their sequences peptides of more conventional length that score well using predictive class-I-binding algorithms, but nevertheless are not reported to induce CTL responses[14]. Moreover, these shorter fragments are often naturally presented on MHC I but fail to be immunogenic[24,29]. Why, in circumstances such as EBV infection of HLA-B35 individuals, these longer immunogenic peptides are presented on MHC I and preferentially selected as targets of CTL is poorly understood. It is most likely the result of biases in antigen processing and T-cell repertoire towards the longer peptide[15,16]. The existence of such epitopes presents new challenges to vaccine design, both in terms of controlling the processing of these long peptides and ensuring the correct conformation is assumed by the epitope that is bound to MHC I.

T-cell recognition of post-translationally modified peptides. T-cell epitopes that have been characterized include glycopeptides; phosphopeptides; deamidated peptides, both through the action of *N*-glycanases and enzymes such as transglutaminase; peptides exhibiting asparagine-bond isomerism, acetylation, methylation, cysteinylation and nitration; and peptides containing disulphide bonds[11,17,30–41]. In addition to modifications that occur spontaneously during ageing, and exposure of proteins to hostile cellular and extracellular environments (for example, oxidation, deamidation), many of these post-translational modifications (PTMs) are performed within or outside the cell by specific enzymes in processes that have complex feedback mechanisms. Breakdown of these feedback mechanisms is frequently associated with pathological processes that result in the accumulation of aberrantly or excessively post-translationally modified material[42–44]. PTMs can be reliably and accurately incorporated into peptides at the time of their synthesis, thereby allowing the end product of complex antigen presentation to be mimicked by a simple compound and the desired immune response to be elicited. Recent reports have described perhaps the most extreme form of PTM, whereby epitopes are generated from discontinuous peptide sequences[45,46] by a protein-splicing mechanism — a mechanism that remains to be fully elucidated. Such processing might occur in a tissue-specific manner and therefore such epitopes are not only impossible to predict but peptide-based vaccines might be the only reliable way of delivering such epitopes to the immune system. Nonetheless, if they are properly characterized they can be easily synthesized for incorporation into subunit vaccines.

Tissue-specific antigen presentation

The proteasome can exist in several different forms depending on the exposure of the cell to pro-inflammatory stimuli[12]. The different forms of the proteasome engender various proteolytic activities and consequently produce an array of peptide precursors for transport into the lumen of the endoplasmic reticulum where they assemble

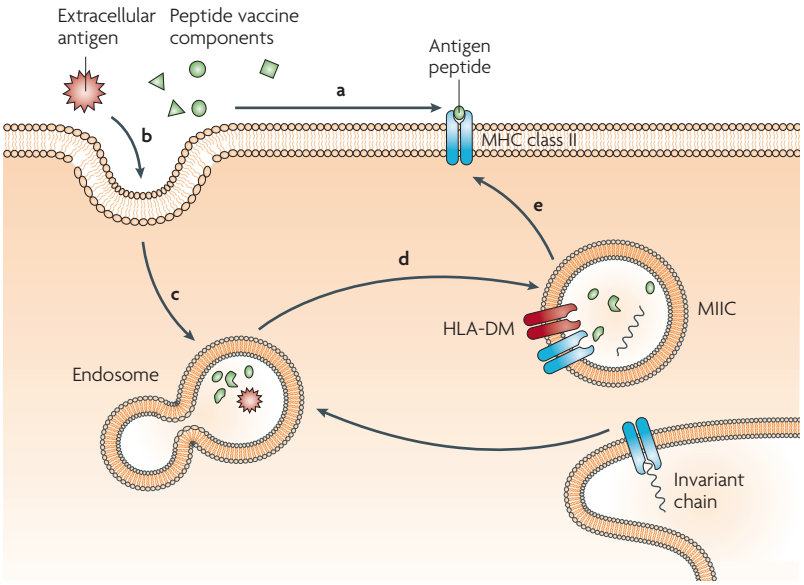


Figure 2 | **The MHC II pathway.** Processing of intact exogenous antigen and peptide vaccines is similar, and both are loaded onto the major histocompatibility complex (MHC) class II molecules. Similar to MHC-I-targeted epitopes, peptide antigens can access surface MHC II molecules and replace existing peptides by a surface-exchange mechanism[113–115] (**a**). Peptide that is administered outside the context of the native antigen will be subjected to an array of cell-surface and extracellular proteases during this process. Exogenous antigen and peptide vaccines are taken up into the endosomal pathway by macropinocytosis or by receptor-mediated events, such as B-cell-surface-immunoglobulin uptake of antigen or Fc-receptor-mediated uptake of immune complexes (**b**). Once the intact antigen or peptide enters the endocytic compartment they are exposed to various proteases, notably the cathepsin family, which are responsible for antigen degradation (**c**). Peptide vaccines therefore need to survive this environment as they are transported to a compartment known as MIIC (a MHC-II-rich endosomal compartment). Here peptides are loaded into MHC II molecules, a process that is facilitated by the chaperone human leukocyte antigen-DM (HLA-DM) that is responsible for removing the invariant (Ii) chain. Another molecule, HLA-DO, also modulates this process in B cells (**d**).

Immunoproteasome
The proteasome is a multisubunit structure found in the cytoplasm, and is responsible for protein degradation. During inflammation some of the constitutive catalytic subunits are replaced by interferon-γ-inducible subunits. This inflammation-induced proteasome is known as the immunoproteasome.

HLA-DM and HLA-DO
These are chaperones encoded by the human major histocompatibility complex, and are responsible for facilitating the peptide loading of human leukocyte antigen (HLA) class II molecules in human antigen-presenting cells such as dendritic cells and B cells.

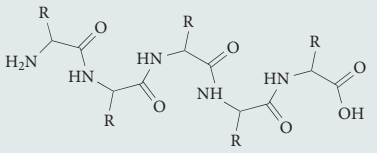
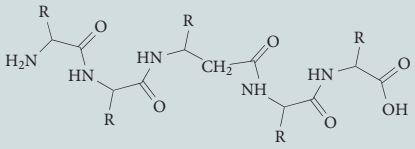
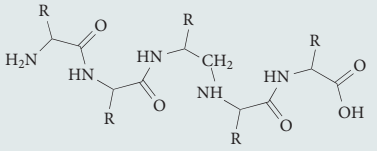
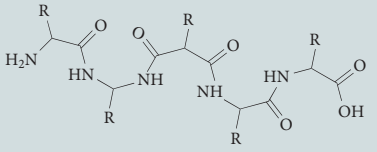
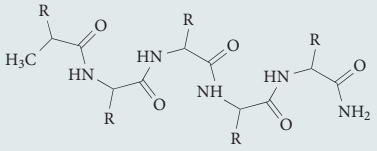
with MHC I. APCs predominantly express the immunoproteasome and potentially present different peptides compared with target tissues, be they infected epithelium or tumour cells that might express the constitutive proteasome without interferon-γ (IFNγ)-inducible subunits. More recently, the interplay of the various forms of the proteasome with other peptidases has been elucidated, adding to the complexity of the processing of antigens to generate the peptides bound to MHC class I molecules that are destined for export to the cell surface[47–49]. This presents a challenge when administering antigens exogenously. To elicit an immune response that mimics natural immunity, the correct, potentially tissue-specific processing events will need to take place to generate immune responses towards epitopes of clinical efficacy. Natural antigen processing also involves the editing of the peptide repertoire by the peptide-editing molecules HLA-DM and HLA-DO. Allelic variation in MHC II molecules can also vary in their dependence on peptide editing by HLA-DM and HLA-DO[50]. A role for tapasin, a putative peptide-editing chaperone involved in the assembly of MHC I with peptide, has also been reported[51–53]. Expression levels of tapasin vary between different tissues, with particularly high levels in activated dendritic cells[54]. Moreover, MHC I molecules also vary in their dependence on tapasin for peptide loading[2,52,55–61]. These features of antigen presentation further complicate the selection of epitopes and prediction of natural immunogenic peptides.

Engineering peptide-based vaccines

Several strategies have been adopted to enhance the effectiveness of peptide therapeutics, including glycosylation, amino-acid-sequence modification, pegylation and cyclization. Of these modalities only the replacement of amino acids within the sequence of a peptide epitope is directly relevant to vaccination studies. Simple epitope modifications have typically consisted of MHC-anchor substitutions in which sub-optimal anchor residues are substituted to improve MHC binding and immunogenicity[62]. In addition, heteroclitic analogues with substitutions outside the MHC-anchor residues have been described that have the capacity to induce hyperstimulation of T cells[63]. These analogues are of interest in the development of vaccines as they can achieve more potent immune responses. This observation might be explained by the increased stability of the MHC/peptide complex, and the increased avidity and residence time of the TCR–peptide/MHC complex[64]. Heteroclitic antigens can also break T-cell tolerance[65–67], which has the potential to assist in the development of antitumour immunity, which frequently involves self-antigens. Although these approaches can produce better vaccine components, they have until recently been constrained to making substitutions using only naturally occurring amino acids[63]. To improve class I binding and TCR avidity, and to introduce favourable biophysical properties to the epitope such as protease resistance and oral stability, it is often desirable to introduce non-natural amino-acid analogues into the peptide epitope.

Several studies have explored modifications that not only provide subtle conformational changes to the peptide/MHC structure, but also incorporate resistance against proteases. The incorporation of β-amino-acids into epitopes can increase the binding affinity of the mimetic for the MHC molecule relative to the wild-type peptide[67–70]. The side chains of β-amino-acids are identical to their parent α-amino-acid, which is of particular importance to maintaining the same properties as the natural epitope. The introduction of a methylene moiety (TABLE 2) into peptides that are solely composed of β-amino-acids results in complete resistance to proteolytic degradation[71]. Furthermore, it has been shown that even single amino-acid substitutions of the naturally occurring α-amino-acid with the homologous β-amino-acid residue can have dramatic effects on the overall stability of the entire peptide[70]. Other methods used to achieve protease resistance and to maintain T-cell cross-reactivity, including peptide-backbone modifications, D-amino-acids or retroinversion of sequences (in which all amino-acids in a epitope are converted to D-amino-acids and the sequence reversed) are summarized in TABLE 2. Modification of the N and C termini of a peptide

Table 2 | **Non-natural amino-acid modifications in peptide vaccines**

Modification	Structure	Refs
Normal α-peptide		
β-amino-acid substitution		68–70
Backbone reduction		116
Partial retro-inversion		116
N-terminal methylation and C-terminal amidation		72

Adapted from REF. 116. R = side chain.

Pegylation
This is the process of attaching one or more chains of polyethylene glycol (also known as PEG) to a peptide molecule, resulting in increased bioavailability and stability.

can also prevent its degradation by exopeptidases[72]. These modifications usually involve *N*-acetylation and *C*-amidation, respectively. The ability to stabilize short peptide epitopes and to control the proteolysis of longer precursor peptides by protecting scissile-peptide bonds or by directing antigen processing allows for the precise delivery of peptide-based therapeutics to the immune system.

Current developments

As discussed before, there is no peptide vaccine on the market yet. However, several promising preclinical and clinical trials for vaccines that involve peptide-based strategies are currently being carried out. For example, recent reports have shown that a multi-epitope approach has been successful for the treatment of hepatitis C viral infections[73], and peptide trials for vaccination in numerous cancers have been reported extensively (see REF. 74 for a review). However, perhaps the most encouraging development is the number of clinical trials supported by both small and large pharmaceutical companies that are based on the use of peptide-based vaccines (TABLE 1a,b). In addition, several new modalities in delivery of vaccines have been developed[75–77] (BOX 2), including the combination of peptide-based and more traditional vaccine approaches. The marriage of epitope engineering and chemical optimization with these new delivery mechanisms promises to provide the next generation of immunotherapeutics.

Future outlook

It is clear that vaccines of the future will require a systematic approach to tailor the desired immune response to individuals. Technology has evolved rapidly to allow for the identification of patient-specific epitopes based on their HLA haplotypes, and these forms of molecular medicines are becoming a reality. Armed with new technologies, immunologists can examine the molecular ingredients of a successful immune response, which have revealed a tremendous amount of biochemical diversity in clinically important epitopes. The use of peptides offers a flexible and simple way to deal with much of this complexity by bypassing the requirements for antigen processing and delivery of a precise and chemically defined payload to the APC. Moreover, peptides display more drug-like properties than recombinant proteins or whole pathogen vaccines, which will open up new avenues for vaccine delivery, and therefore attract increasing interest from the pharmaceutical industry.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
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Laboratory of James McCluskey: http://www.microbiol.unimelb.edu.au/research/groups/mccluskey
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