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Peptides, Proteins & Antisense

Ii-Key/MHC class II epitope hybrids: a strategy that enhances MHC class II epitope loading to create more potent peptide vaccines

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Life-threatening diseases, such as cancer and pandemic influenza, demand new efforts towards effective vaccine design. Peptides represent a simple, safe and adaptable basis for vaccine development; however, the potency of peptide vaccines is insufficient in most cases for significant therapeutic efficacy. Several methods, such as Ligand Epitope Antigen Presentation System and ISCOMATRIX®, have been developed to enhance the potency of peptide vaccines. One way of increasing the loading of MHC class II peptides occurs through the use of Ii-Key technology. Ii-Key (LRMK), a portion of the MHC class II-associated invariant chain (Ii), facilitates the direct loading of epitopes to the MHC class II molecule groove. Linking the Ii-Key moiety via a simple polymethylene bridge to an MHC class II epitope, to generate an Ii-Key/MHC class II epitope hybrid, greatly enhances the vaccine potency of the tethered epitope. The combination of such Ii-Key/MHC class II epitope hybrids with MHC class I epitope-containing peptides might generate a potent peptide vaccine for malignancies and infectious diseases. The Ii-Key hybrid technology is compared with other methods that enhance the potency of a peptide vaccine.

Keywords: allosteric site, antigen presentation, Ii-Key hybrid, immunotherapy, MHC class II, vaccine

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1. Introduction

The recent outbreak of severe acute respiratory syndrome and the threat of a pandemic by the highly pathogenic avian influenza virus in Asia demand the rapid development of potent vaccines. This type of vaccine development technology could also greatly benefit tumour immunotherapy, but will require more potent vaccine strategies. Peptides represent the safest form of all vaccine modalities, as they are comprised of the minimal elements required for generation of an effective immune response: MHC class I and/or class II epitopes. However, although immune responses have been observed using peptide vaccines, the demonstration of clinical efficacy is rare, pointing to the need for increased potency. Although peptide vaccine research initially focused on MHC class I epitopes to induce cytotoxic T lymphocyte (CTL) activity, MHC class II epitope vaccines for the induction of T helper (Th) cell activity have drawn growing attention [1-4]. Recent data have clearly shown that CD4+ Th cell activation is required for the induction of a potent immune response against an immunogen. Antigen-specific Th cells are needed for full activation of antigen-specific CD8+ CTLs and to provide long-term antigen-specific memory [5-7].

A variety of methods have been explored towards the goal of enhancing the potency of peptide vaccines. A novel technology based on using a portion of the MHC class II-associated invariant chain (Ii) to enhance MHC class II epitope charging and, thus, the efficiency of Th cell activation has been developed [8-11]. This segment of the Ii, termed 'Ii-Key', significantly enhances MHC class II epitope presentation in a variety of settings and creates a practical method to enhance the efficacy of MHC class II peptide vaccines. The Ii-Key segment does this by binding to an allosteric site on MHC class II molecules to loosen their epitope-binding groove, allowing the epitope segment to directly charge MHC class II molecules present on the cell surface [9,10]. Ii-Key hybrids are composed of the Ii-Key moiety linked via a simple polymethylene bridge to the N-terminus of an MHC class II epitope. The authors review data showing that Ii-Key hybrids are much more potent than epitope-only peptides, both *in vitro* and *in vivo*, when used in conjunction with epitopes relevant to different diseases [12-16]. Ahlers *et al.* [17] showed that enhanced activity of MHC class II epitopes, rather than modification of MHC class I epitopes, plays a more significant role in the optimal enhancement of a CTL immune response. This finding supports a strategy for the combined use of Ii-Key/MHC class II hybrids with MHC class I epitope peptides to generate potent peptide vaccines for malignant and infectious diseases.

2. MHC class II antigen presentation and CD4+ T cell activation in tumour immunity and virus infection

2.1 MHC class II antigen presentation and function of the Ii protein

Th cell activation by MHC class II-presented antigens is essential for the immune system to mount an effective and long-lasting response [5-7]. The initial step towardss activating CD4+ Th cells is for the antigen to be acquired by professional antigen-presenting cells (APCs) and processed for presentation by MHC class II molecules. Such Th cells then help either directly through the activation of CD8+ CTLs or indirectly through licensing of dendritic cells (DCs), which in turn can optimally stimulate the activation and development of CD8+ CTLs [18,19]. A complete immune response to malignant or infectious diseases includes the activation of both CD4+ Th cells and CD8+ CTLs by MHC class II and class I presentation, respectively.

The Ii protein normally binds to MHC class II molecules in the endoplasmic reticulum (ER) at synthesis and protects the epitope-binding site on MHC class II molecules from binding to endogenously derived epitopes in the ER, as normally occurs with MHC class I molecules [20,21]. Another major function of the Ii protein is to enhance exogenous peptide charging to MHC class II molecules [22-24]. The MHC class II/Ii complex is transported to a post-Golgi, antigenic peptide-binding compartment after synthesis [25-27]. In such compartments, the Ii protein is cleaved by proteases to allow charging by exogenously derived epitopes. After being charged with epitopes, the MHC class II–epitope complex travels to the cell surface for presentation to CD4+ Th cells [28-31]. Two mechanisms have been proposed to explain the function of the Ii protein in enhancing the charging of epitopes to MHC class II molecules. First, the Ii protein is partially digested to leave only a small segment behind, termed class II-associated invariant chain peptide (CLIP), which is bound to the epitope-binding groove of the MHC class II molecule in a manner to keep the groove open [32-34]. HLA-DM then exchanges CLIP for an epitope [35,36]. Second, in a concerted manner, Ii protein is digested and released from MHC class II molecules as the epitopes are being charged [22-24]. An important function of the Ii is evident in a short sequence that binds to an allosteric site outside of the epitope-binding groove [10]. The result of this interaction is to keep the epitope-binding groove in a conformation that is the most suitable for MHC class II molecules to exchange an epitope. That Ii sequence has been termed the Ii-Key peptide, the discovery and optimisation of which is discussed in later sections [8-11].

2.2 Significance of CD4+ T cell activation in antitumour immunity and virus infection

CD4+ Th cells play a critical role by inducing and maintaining both CD8+ T and B cell responses, and in maintaining immunological memory [5-7,37-40]. For example, Ossendorp *et al.* [41] established that tumour antigen-specific Th cells are required for optimal induction of CTLs against MHC class II-negative tumours. The role of CD4+ Th cells in cancer immunity is further highlighted by significant clinical results obtained in melanoma patients receiving adoptive transfer of highly reactive CD8+ and CD4+ T cells [42,43]. Gao *et al.* [44] showed that in order to activate memory CD8+ T cells to become fully functional tumour killer cells, antigen-specific CD4+ Th cells are required. Yu *et al.* [45] defined how the complementary role of CD4+ Th cells is required for efficient cross-presentation of tumour antigens to CD8+ T cells. Furthermore, CD4+ T cells can help to break down tolerance to persistent self-antigens (e.g., tumour-associated antigens) to fight established tumours in an IL-2-dependent mechanism [46]. Along with the continuing discovery of novel defined epitopes, the investigation of MHC class II epitope-based vaccines in tumour immunotherapy is advancing [1-4,47-49].

CD4+ T cell activation is also recognised as essential to obtaining potent CTL responses, optimal antibody responses and immunological memory in virus infection [50-55]. Kamal *et al.* [50] demonstrated that long-lasting HCV clearance requires vigorous, up-front and strong CD4+ T cell responses. Patke and Farber [51] showed that optimal long-term immunity depends on the functional persistence of CD4+ memory T cells. Using an influenza virus nucleoprotein immunisation model, Johansen *et al.* illustrated that CD4+ T cells are essential for the differentiation of CD8+

memory T cells. In addition, $CD8^+$ memory cells generated with this type of help survived better in secondary polyclonal hosts [53]. Th cells can create immunological memory for some viruses for 10 – 30 years, as well as enhance CTL activity [56,57]. Moreover, DCs acquire 'licensing' from antigen-specific $CD4^+$ T cells before they can activate and expand $CD8^+$ CTLs [18,19]. Influenza virus-specific $CD4^+$ T cells direct $CD8^+$ responses by secreting a Th1 panel of cytokines that drive B cells to produce neutralising antibodies and generate memory B cells [58,59]. Ahmadzadeh and Farber [60] showed that $CD4^+$ T cells can also be direct effector cells in responding to influenza antigens (by secreting cytokines), as well as contributing to immunological memory. Furthermore, influenza virosomes enhance MHC class I-restricted CTL induction through $CD4^+$ Th cell activation [61]. Schmitz *et al.* showed increased mortality of IL-1 receptor-deficient ($IL\text{-}1R1^{-/-}$) mice together with the defective recruitment of $CD4^+$ T cells to the site of infection [62]. Similarly, Van der Sluijs *et al.* showed that $IL\text{-}18^{-/-}$ mice had a reduced clearance of the virus, and that this finding was related to the reduced $CD4^+$ T cell activation in the lung [63]. Thus, good preactivation of virus-specific $CD4^+$ T cells is valuable in both reducing the incidence of influenza infection in a large population, wherein people may be exposed to low doses of virus, and in greatly decreasing the severity of symptoms of influenza in those who might be exposed to high doses of the virus.

3. The activity of Ii-Key and Ii-Key/MHC class II epitope hybrids

3.1 Identification of the core Ii-Key sequence

The segment of the Ii-containing amino acids hIi(77-92) regulates tightness of closure of the antigenic epitope-binding groove of MHC class II molecules. This segment first raised interest as it has six positive side chains, no negative side chains and four prolines, which together appeared to constitute a signal for a protease or 'exchange-ase'; ostensibly to regulate cleavage and release of the Ii (**Figure 1**) [9,64]. Further studies showed that mutations in this segment do, in fact, block the staged cleavage and release of Ii [22,23]. In light of these findings, the fragment of the Ii-containing amino acids hIi(77-92), referred to as 'Ii-Key' was synthesised. An initial study illustrated that the activation of hen egg lysozyme-specific T cell hybridoma by an hen egg lysozyme epitope peptide was enhanced by Ii-Key peptides ≥ 50-fold [8]. Enhanced activation was observed even when using paraformaldehyde-fixed APCs, in which normal intracellular processing was not possible. In order to further identify the minimal active sequence of Ii-Key, C- and N-terminal deletions, as well as amino acid substitutions, were created [9]. In all, the activities of 160 homologues of the Ii 77 – 92 sequence were characterised in the same murine T hybridoma activation assays. Those studies revealed a 'core' LRMKLPK structure that had greater potency than the original 16-amino acid peptide. Even the Ii 77-80(LRMK) segment retained ≥ 50% of the activity of LRMKLPK. For simplicity, therefore, a Ii-Key/MHC class II epitope hybrids with this shorter Ii-Key moiety was later designed.

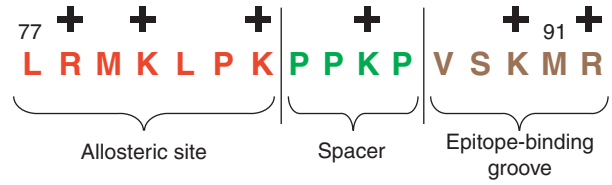

Figure 1. Identification of Ii-Key. The Ii protein region 77 – 92 has six positively charged amino acids, four prolines and no negatively charged amino acids, suggesting a site for proteolytic or exchange-ase activity. The segments interacting with the allosteric site and epitope-binding trough are indicated, as well as an intervening spacer region.

3.2 Activity of Ii-Key hybrids *in vitro* and *in vivo*

In contrast to cell culture studies, *in vivo* inoculation of mice with Ii-Key plus an antigenic peptide failed to enhance the activity of that antigenic peptide (unpublished observations). This suggested that the Ii-Key needed to be colocalised with the antigenic epitope to enhance presentation. The Ii-Key moiety was linked covalently to the MHC class II epitope to ensure that individual MHC class II molecules on the APC are exposed simultaneously to both Ii-Key and epitope. A systematic series of Ii-Key MHC class II epitope hybrids were synthesised and tested in an *in vitro* T cell hybridoma stimulation assay [12]. For this series of hybrids, the Ii-Key core (LRMK) was joined to an MHC class II-restricted epitope of pigeon cytochrome c (PGCC81-104). The spacers joining Ii-Key and PGCC81-104 were either a simple polymethylene (δ-aminovaleric acid) linker or the natural sequence of the Ii extending from the C terminus of LRMK. The design of Ii-Key hybrids was based on biochemical and X-ray crystallographic data indicating that the Ii-Key binding site lies outside of the antigenic peptide-binding groove of MHC class II molecules [65]. Both the length of the Ii-Key derivative and linker composition were varied within the series. Hybrids having either type of bridge were effective. Some hybrids enhanced presentation of an antigenic epitope up to 250 times above the baseline stimulation observed using the free antigenic peptide [12].

To assess the *in vivo* activity of Ii-Key/MHC class II epitope hybrids using a clinically relevant epitope, an MHC class II-restricted epitope from the human melanoma associated antigen gp100(46 – 58) [66] was used to design a series of hybrids. Ii-Key (LRMK) was linked via δ-aminovaleric acid to amino acids that were -3, -2 and -1 residues N-terminal to the amino acid predicted to occupy the P1 site of the epitope-binding groove. Immunising HLA-DR4-IE transgenic mice with this series of

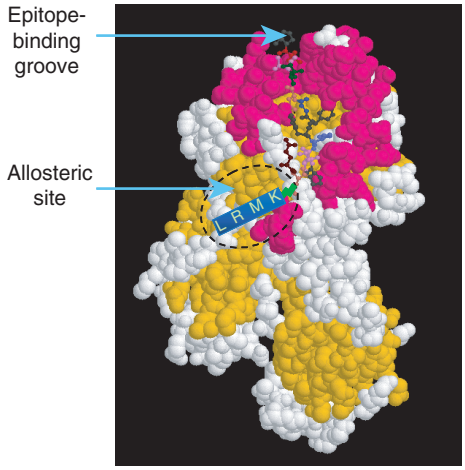

Figure 2. Diagram of the relationship of the epitope binding groove (top arrow) and allosteric site (lower arrow) on MHC class II molecules. The epitope binding groove is seen on angle. The dashed oval area is the proposed allosteric site. Ii-Key (LRMK) binds to the allosteric site to regulate conformational changes of MHC class II molecule.

Ii-Key/gp100 hybrids showed that the Ii-Key hybrid in which LRMK is linked via δ-aminovaleric acid directly to the amino acid occupying the P1 site gave the highest activity in an enzyme-linked immunospot (ELISPOT) assay of splenocytes or purified CD4+ T lymphocytes [16]. This indicated that the spacer length between the Ii-Key moiety and the MHC class II epitope can influence activity. Similarly, generation of a higher titre of gp100(46 – 58)-specific antibodies was also induced by an Ii-Key/gp100 hybrid compared with an epitope-only peptide [15]. There was no evidence that Ii-Key hybrids skew preferentially towards Th1 or 2 response [15,16]. Similar *in vivo* results were obtained with Ii-Key/HIV gp160(843 – 852) and Ii-Key/HIV gag(279 – 292) hybrids [14]. The activity of hybrids was stronger in mice compared with those receiving epitope-only peptides, as measured in IL-4 and IFN-γ ELISPOT assays of splenic lymphocytes using epitope-only peptides.

In order to assess the activity of Ii-Key hybrids in human cells, an Ii-Key/HER-2/neu(777-789) epitope hybrid was used to stimulate lymphocytes from both a healthy donor and a patient with HER-2/neu-positive metastatic breast carcinoma. The *in vitro* proliferation and IFN-γ release was more strongly stimulated by the Ii-Key hybrid than by the epitope-only peptide [13]. Subsequent studies, using the peripheral blood mononuclear cells from > 10 patients with HER-2/neu-positive cancer, have confirmed the increased Th activity of Ii-Key/HER-2/neu hybrids relative to epitope-only peptide in boosting CTL effectors (Sotiriadou NN *et al.*, manuscript submitted).

4. MHC class II allosteric site and the molecular mechanism underlying Ii-Key enhancement of MHC class II epitope activity

To further elaborate the molecular mechanisms underlying Ii-Key enhancement of epitope binding to MHC class II molecules, soluble recombinant HLA-DR1 was used to characterise the result of physical interactions between Ii-Key containing peptides and MHC class II molecules [10]. The binding or release of biotinylated hMBP(90 – 102) by Ii-Key was quantified by measuring the interaction with streptavidin. All Ii-Key peptides examined facilitated the binding or release of the antigenic peptide hMBP(90 – 102) from MHC class II molecules. Some Ii-Key peptides with long C-terminal extensions from the 7 amino acid core LRMKLPK sequence effectively released bound antigenic peptide in the absence of free peptide in the solution, indicating that longer Ii-Key C-terminal extensions might reach the N-terminus of the epitope-binding groove to kick bound peptides out. Further competitive binding experiments using biotinylated Ii-Key peptides and hMBP(90-102) helped to define the spatial relationship of the allosteric site relative to the antigenic peptide-binding site [10]. Those studies indicated that the allosteric site is located a few amino acids away from the N-terminal end of the epitope-binding groove (**Figure 2**). A similar allosteric effect on MHC class II molecules was described by Gerlier *et al.* [67], who summarised evidence that a second peptide could bind transiently to MHC-II molecules outside the groove and have an allosteric effect on peptide–MHC-II complex formation.

A model for the regulation of binding by the Ii-Key moiety of Ii-Key/class II epitope hybrids through interaction at the allosteric site of MHC class II molecules is presented in **Figure 2**. Initially, binding of the Ii-Key moiety to the allosteric site induces a conformational change in the antigenic epitope-binding groove such that it becomes more accessible for MHC class II epitope loading. After the epitope portion of the hybrid has bound to the MHC class II molecule, the Ii-Key dissociates to allow stabilisation of the MHC class II–epitope complex. Alternatively, the binding of the antigenic epitope may pull the Ii-Key motif away from the allosteric site to stabilise epitope binding. As a result, the efficiency of an epitope linked to Ii-Key to directly bind to MHC class II molecules for presentation to CD4+ T cells, is higher.

Studies complementary to those cited below also provide data indicating the existence of an allosteric region on MHC class II molecules. Feng and Lai [68] showed that a low-affinity peptide with hydrophobic and cationic side chains enhanced activation of a T cell clone by the indicator antigen. Tampe and McConnell [69] showed in fluorescence energy transfer studies that two peptides could bind simultaneously in close proximity on MHC class II molecules, suggesting the presence of a distinct

peptide-binding region near the epitope-binding groove. Peptides interacting with the second site enhanced the rate for binding of an antigenic peptide [70,71]. With the identification of the Ii-Key allosteric site as described in this review, studies on the influence of 'second peptides' on the binding of antigenic peptides to MHC class II molecules can be seen in a new light. Additional early studies pointing to the likelihood of an allosteric region came from studies with CLIP. Urban and Strominger [72] found that the ability of CLIP to bind to MHC class II molecules under acidic conditions depended on the presence of N-terminal amino acid residues from positions 81 – 89. Kropshofer *et al.* [73] determined that at pH 5.8 CLIP Ii90-105 dissociates rapidly from HLA-DR3 molecules, but only when the L^{81}PKPPKPVS^{89} is present. These results indicated that the N-terminus of CLIP may have an allosteric effect on the binding of CLIP Ii90-105. Similarly, Stumptner and Benaroch [74] (under different experimental conditions) demonstrated that the Ii81-90 sequence loosens the association of CLIP81-104 with MHC class II molecules. Bishof *et al.* [75] showed that infusion of mice with a recombinant Ii in which CLIP was replaced by a myelin basic protein (MBP) epitope 84 – 96, to generate Ii-MBP84-96, was more effective than MBP84-96 epitope-only peptide when used either as an activating or suppressing immunogen in a murine model of experimental allergic encephalomyelitis. One explanation is that the Ii-MBP84-96 recombinant protein, which contains both the Ii-Key sequence and the MBP MHC class II epitope (84 – 96), interacts with the allosteric region of class II molecules to enhance epitope presentation. These studies indicated that the allosteric site lay at the distal end of an allosteric region, and that binding of Ii-Key to it significantly altered the conformation of MHC class II molecules [8-10,12]. Another line of evidence comes from biological function assays. Hess *et al.* [76,77] have defined that the N-terminal segment of CLIP can augment the immunogenicity of cryptic 'self' tumour-associated antigens. A chimeric construct of an MHC class II binding peptide from the *c-erb* oncogene (HER-2/neu) containing the N-terminal flanking region of CLIP elicited potent antitumour activity against a HER-2/neu-positive tumour in a rat model system. However, the N-terminal sequence was directly linked to the HER-2/neu epitope and it is possible that a new MHC class II epitope was created for the enhancement of the immunogenecity of the HER-2/neu epitope. Although the N-terminal sequence portion of the chimeric peptide may bind at another portion of a larger allosteric region, it is unlikely to bind to the Ii-Key site.

The discovery of Ii-Key is of significant importance in designing potent peptide vaccines. The MHC class II–epitope complex is relatively stable [78,79]. Without Ii-Key, peptide epitopes have difficulty displacing the prebound ambient peptides on MHC class II molecules at the cell surface. With the help of Ii-Key, however, the peptide-binding groove on MHC class II molecules can be opened and closed easily, offering an efficient method to enhance the binding of vaccine peptides to MHC class II molecules.

5. Other methods that enhance the potency of peptide vaccines

A variety of techniques have been explored to improve the activity of peptide vaccines, but none are MHC class II epitope-specific. These methods use different mechanisms for peptide vaccine enhancement. Improving the delivery of MHC class II epitopes into APCs is a common approach to enhancing MHC class II peptide vaccines. For example, exosomes are used as a delivery vehicle for better activation of both $CD8^+$ and $CD4^+$ T cells [80]. Another method is ISCOMATRIX®, which is a cage-like structure composed of antigens, such as peptides and adjuvants [81]. ISCOMATRIX effectively induces both humoral and cellular immune responses against the antigens incorporated in the structure by enhancing the delivery of peptide antigens and by providing adjuvant stimulation. APCs usually have difficulty acquiring soluble antigen. Antigen–antibody complexes are more accessible to APCs through the recognition of the Fcγ receptor on APCs by the Fc domain on an antibody [82]. Molecular chaperones are necessary components for better binding of epitopes to MHC class II molecules. Bacterial HSP 70 enhances the immune response against MHC class II epitopes complexed to bacterial HSP70 [83]; however, the enhancement occurs only at low pH, indicating that chaperone-complexed epitope binding to MHC class II is also limited by CLIP [72]. Recently, peptide conjugates targeting specific components of immune cells have been investigated. An example of the latter employs a T cell-binding ligand coupled to a peptide antigen (Ligand Epitope Antigen Presentation System [LEAPS]) [84]. As Ii-Key hybrids target the charging of MHC class II molecules, antigen presentation to T cells is more selective than LEAPS technology. Peptide vaccines are usually limited by polymorphic MHC class II allele restrictions. A nonspecific Th epitope technology, the pan-DR epitope (PADRE), has also been developed to circumvent this limitation [85]. The advantage of PADRE is that it overcomes restrictions by HLA-DR alleles. DNA vaccines using the Ii gene as a delivery carrier to deliver Th epitope to MHC class II molecules has been developed [86]. This method utilises an Ii gene in which the CLIP portion has been replaced by a DNA fragment encoding a MHC class II epitope. This method has successfully induced epitope-specific $CD4^+$ T cells. Lastly, it should be noted that Ii-Key hybrid technology is compatible with many of the methods discussed to further enhance the overall antigen-specific response. For example, Ii-Key hybrid peptides might be incorporated into ISCOMATRIX to further enhance the potency of the vaccine.

6. Clinical advantages of Ii-Key immunotherapy

6.1 Need for peptide vaccine potency to breakdown tolerance to tumour antigens

The discovery of numerous clinically relevant peptide epitopes, both MHC class I- and II-restricted, has increased the motivation to develop effective peptide vaccines. From these data, consensus motifs for both MHC class I- and II-restricted epitopes have been proposed [87-89]. Although some peptides of potential clinical importance have been identified and specific immune responses have been observed in patients treated with those peptides, good therapeutic efficacy has not been observed [90-93]. The main obstacle appears to be the relatively low affinity of some MHC class II-restricted epitopes. By enhancing the ability of peptide epitopes to charge MHC class II molecules directly on the cell surface, the Ii-Key hybrid technology opens the door to a potent and clinically practical strategy for peptide immunotherapy.

Another challenge specific to cancer immunotherapy is that tumour antigens are usually tolerated as self by the immune system; therefore, the main task of clinical immunologists is to breakdown tolerance to specific, tumour-associated self-antigens [66,90-93]. The authors propose that the ability of Ii-Key hybrids to enhance activation of Th1 $CD4^+$ cells will help to break tolerance to tumour antigens. In the authors' *in vivo* and *in vitro* studies using Ii-Key/gp100(46-58) and Ii-Key/HER-2/neu(777-789) hybrids [15,94], significantly stronger $CD4^+$ T cell activity was obtained over the native peptides. Furthermore, the use of Ii-Key hybrids with inflammatory cytokines or adjuvants is expected to enhance the activity of hybrids and likewise help to breakdown tolerance against tumour antigens.

6.2 Ii-Key hybrid and HLA-DR restriction

MHC class I and class II allele restriction represents another obstacle for peptide vaccine design. Ii-Key/MHC class II epitope hybrids may have the potential to mitigate this problem. Higher-affinity epitopes are generally more tightly restricted to a specific MHC class II allele, whereas medium-affinity epitopes are usually more promiscuous [101,102]. However, $CD4^+$ Th cells specific for medium-affinity epitopes are usually overwhelmed by $CD4^+$ Th cells specific for high-affinity epitopes. Ii-Key hybridisation increases the binding of an epitope to MHC class II molecules and, thus, increases the binding activity of medium-affinity epitopes. That is, Ii-Key has the potential of turning a medium-affinity epitope into a high-affinity epitope while retaining the promiscuous properties of the medium-affinity epitope. High-affinity MHC class II epitopes shared among the existing influenza vaccines that might be useful in priming Th cells against the potentially pandemic H5N1 strain have been examined. No common top-scored MHC class II-restricted haemagglutinin epitopes were found from three strains of influenza virus examined (A/New Caledonia/20/99, A/Wyoming/03/2003 and B/Jiangsu/10/2003). The conclusion is that there can be no Th cell cross-protection to H5 using class II epitopes targeting H1 strains (i.e., antigenic shift); however, vaccination with Ii-Key hybrids incorporating medium-affinity epitopes might offer cross-protection between different H5 stains (i.e., antigenic drift). In this manner, Ii-Key may enhance the ability to use 'promiscuous' MHC class II epitopes for preventive vaccination and/or immunotherapy.

6.3 Clinical use of Ii-Key hybrids: combination with CTL epitopes, DNA vaccine or recombinant protein

As mentioned previously, it is clear that the induction of a complete immune response requires activation of both $CD4^+$ Th cells and $CD8^+$ CTLs. This is particularly true for tumour immunotherapy. Although Ii-Key/MHC class II hybrid vaccines can induce long-term, antigen-specific $CD4^+$ T cell stimulation, the induction of CTL activity requires the addition of MHC class I epitopes. As a number of MHC class I peptide vaccine trials for melanoma have been shown to be ineffective in the clinic, investigators are now focusing on the concomitant activation of $CD4^+$ T cells by adding MHC class II epitopes to the vaccine formulation [2-4,91,95. MHC class I epitopes can be simply mixed with Ii-Key hybrids or linked to the C-terminus of the Ii-Key hybrid to form a double hybrid (**Figure 3**). Theoretically, one Ii-Key hybrid could be linked to multiple MHC class I epitopes to increase the coverage of CTL specificity [96]. Alternatively, Ii-Key hybrids could be used together with a DNA vaccine. The major advantage of DNA vaccines is that they induce a strong CTL response. Different prime–boost regimes using DNA–virus, DNA–protein and DNA–peptide have been developed in an effort to enhance the activity of DNA vaccines [97-99]. A DNA/Ii-Key hybrid or Ii-Key hybrid/DNA prime–boost regimen (DNA for the induction of $CD8^+$ CTLs and Ii-Key for the induction of $CD4^+$ Th cells) could be a very effective regimen for inducing a robust immune response both for tumour immunotherapy and in vaccination against viral infection. Lastly, Ii-Key hybrid vaccines may be effective alone by generating a potent epitope-specific Th1 $CD4^+$ T cells against viral infection. For example, during acute HCV infection, patients with strong $CD4^+$ T cell responses clear the virus, whereas patients unable to mount specific Th1 immunity develop chronic disease [51,52,100]. Pre-existence of virus epitope-specific $CD4^+$ Th1 memory cells, induced by Ii-Key hybrid vaccines, could eventually prevent a large population from being infected by HCV virus at lower levels of exposure, and greatly reduce the symptoms in those who may be exposed to high levels of the virus.

7. Expert opinion and conclusion

Potent Ii-Key/MHC class II hybrid vaccines can be created by covalently linking the N-terminus of an MHC class II epitope through a polymethylene bridge to the C-terminus of the Ii-Key segment of the Ii. Such Ii-Key hybrids significantly

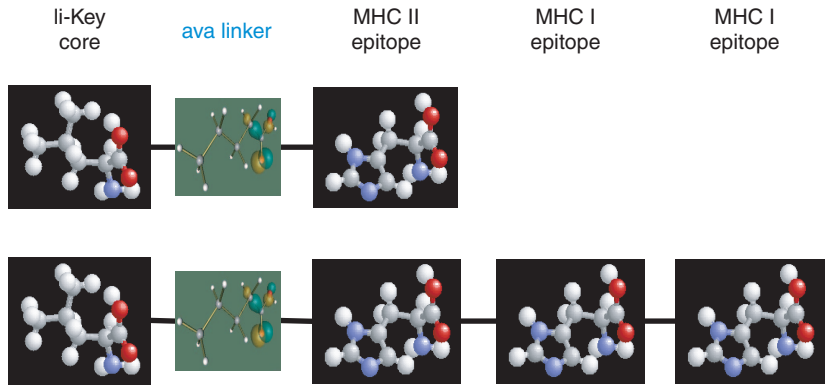

Figure 3. Diagram of Ii-Key hybrid and Ii-Key double hybrid. An Ii-Key hybrid is composed of an Ii-Key core (LRMK), a δ-aminovaleric acid linker and a MHC class II epitope. One or more MHC class I epitopes can be attached to the C-terminus of the Ii-Key hybrid, forming a double hybrid.

enhance the potency of MHC class II epitope vaccines in activating epitope-specific $CD4^+$ Th cells *in vitro* and *in vivo*. Enhanced Th cell activation afforded by hybridisation with Ii-Key represents an important advance in the design of peptide vaccines. The use of Ii-Key/MHC class II hybrids makes it possible to envision a collection of 3 – 5 Ii-Key/MHC class II epitope hybrids restricted to HLA-DR alleles present in > 90% of the population to be used for the induction of $CD4^+$ T cells against critical viral infections. The pre-existence of MHC class II epitope-specific $CD4^+$ memory cells (induced by an Ii-Key hybrid vaccine) may prevent a large population from being infected by low-dose exposure to pandemic influenza, and greatly reduce the symptoms in those who may be exposed to high doses of the influenza virus. Ii-Key/MHC class II hybrid vaccines could also be used together with a DNA vaccine encoding the same antigen from which the MHC class II epitope was obtained for tumour immunotherapy. Likewise, Ii-Key/MHC class II hybrids can be used with a collection of MHC class I CTL epitopes from the same tumour antigen to provide both $CD4^+$ and $CD8^+$ T cell stimulation. Finally, the antigen-specific mechanism of Th stimulation allows Ii-Key hybrid technology to be used together with other strategies to further enhance the potency of the MHC class II vaccine peptides.

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