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Ii-Key/MHC class II epitope peptides as helper T cell vaccines for cancer and infectious disease

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1. ABSTRACT

Potent MHC class II antigenic peptide vaccines are created by covalently linking the N-terminus of a MHC class II epitope through a polymethylene bridge to the C-terminus of the Ii-Key segment of the Ii protein. Such hybrids enhance potency of presentation *in vitro* of the MHC class II epitope about 200 times relative to the epitope-only peptide. *In vivo*, as measured by IFN-γ ELISPOT assays, the helper T cell response to vaccination is enhanced up to 8 times. The design of such hybrid vaccine peptides comes from insight into the mechanism of action of the Ii-Key motif within the Ii protein, in regulating antigenic peptide binding into the antigenic peptide binding groove of MHC class II molecules. Here we present the logic and experimental history of the development of these vaccine peptides, with particular attention to the hypothesized mechanism of action. Methods for the design and testing of these peptides are presented. Experience in developing peptide vaccines for immunotherapy of cancer is reviewed, focusing on the clinical potential of Ii-Key/MHC class II epitope hybrids.

2. INTRODUCTION

Improving immune responses of helper T cells to tumor antigens by vaccinating with peptides from those antigens containing MHC class II epitopes could enable curative immunotherapies for many cancers. Stronger helper T cell responses induce better cytotoxic T lymphocytes (CTL), antibody responses, and immunological memory. Today, vaccinating with peptides containing MHC class II epitopes is not very effective clinically compared to vaccinating with MHC class I epitope-containing peptides. MHC class II peptides have tight binding affinities for cell surface MHC class I molecules, for presentation to CTL. In contrast, MHC class II peptides much be present at much higher concentrations to become substituted into cell surface MHC class II molecules for presentation to helper T cells. Our discovery centers on a method to overcome the weakness of presentation of MHC class II epitope vaccine peptides.

MHC class II epitopes are normally bound to MHC class II molecules in a post-Golgi, antigenic peptide

binding compartment of antigen presenting cells (dendritic cells, macrophages and B cells). The Ii protein, which is coded by only one gene in all humans, forms a trimer at synthesis in the endoplasmic reticulum. Three dimers of MHC class II α and β chains are bound to that trimer, and the complex is transported intracellularly to the antigenic peptide-charging compartment. There the Ii protein is cleaved by proteases, as are the internalized antigens, and antigenic peptides are bound into the antigenic peptide binding site. The CLIP (cleaved leupeptin-induced peptide) peptide of the Ii protein remains on some MHC class II molecules and might facilitate charging of MHC class II molecules with antigenic peptides.

A second immunoregulatory peptide, called the Ii-Key peptide, also regulates antigenic peptide binding and release of antigenic peptides, even on cell surface-expressed MHC class II molecules. Linking an Ii-Key core sequence covalently through a simple polymethylene spacer to an antigenic epitope creates potent Ii-Key/MHC class II epitope hybrid peptide vaccines. For example, linking the Ii-Key moiety LRMK to the melanoma gp100(48-58) MHC class II epitope significantly enhances the vaccine response to that epitope in DR4-IE transgenic mice. In ELISPOT assays on splenic lymphocytes of those mice both the number of responding cells and cytokine output per cell are increased. Frequently, the most effective vaccine hybrid is the one with a shorter linker between Ii-Key and the epitope. Mechanistic reasons for this observation are considered. Ii-Key/MHC class II epitope hybrid peptide vaccines induce strong Th1 responses to various MHC class II epitopes from HER-2/neu, melanoma gp 100 and tyrosinase, HIV gp120, Gag and Nef, and influenza H5 hemagglutinin. Such hybrids can be applied to many diagnostic and therapeutic uses for cancer and infectious diseases, and to modify allergy or autoimmune disease.

3. DISCOVERY OF THE FUNCTION OF THE Ii-KEY SEGMENT OF THE Ii PROTEIN TO REGULATE TIGHTNESS OF BINDING OF ANTIGENIC PEPTIDES INTO MHC CLASS II MOLECULES

The characterization of the biological function of the Ii-Key segment of the Ii protein to regulate antigenic peptide charging into MHC class II molecules occurred in stages over many years. From the initial hypothesis that Ii protein blocked the MHC class II binding site (1) many experiments were executed to characterize the changing structure of Ii protein in association with MHC class II molecules (2-7). In particular, cleavage and release of Ii fragments (8-10) led to experiments to determine whether antigenic peptides are bound in a concerted fashion with the release of Ii peptide from the MHC class II molecules (11). An unusual sequence of Ii protein was evaluated as a signal to other molecules to regulate the cleavage and/or peptide charging events (12). Almost by accident, the function of that peptide was discovered to enhance charging of antigenic peptides to MHC class II molecules (13). Recent progress has focused on optimal design and clinical application of the hybrid, joining the core of the Ii-Key segment through a polymethylene bridge to the N-terminus of MHC class II antigenic epitopes (14). The continuing questions focus on optimizing structure of such hybrids, especially when the peptide with the antigenic epitope contains more than one closely overlapping epitopes. In addition, we are examining the role of the hybrids in enhancing selectively Th1 or Th2 responses, and approaches toward breaking tolerance in cancer patients.

3.1. Logic and experimentation leading to potent Ii-Key/MHC class II epitope hybrid vaccine peptides

A novel mechanism for boosting responses to MHC class II epitope vaccine peptides exploits a MHC class II molecule regulatory allosteric site, which governs tightness of binding of MHC class II epitope peptides. The normal process of MHC class II antigen charging and presentation is highly controlled, in order to assure fidelity in presentation of selected peptides. The Ii protein associates with MHC class II molecules at synthesis in the endoplasmic reticulum and prevents their charging with endogenous peptides, which are otherwise destined for binding to MHC class I molecules. After the MHC class II molecule complexes have been transported into a post-Golgi antigenic peptide-charging compartment, the Ii protein is digested away allowing exogenous peptides to bind into the antigenic peptide-binding site of MHC class II molecules (10, 11, 15, 16).

A critical role of the Ii-Key motif in the Ii protein is during the process of Ii protein digestion and in the concerted release of fragments from the MHC class II molecules and antigenic peptide charging. The binding of radiolabeled, photo-crosslinking, antigenic peptides to MHC class II molecules is more efficient during the cleavage and release of the Ii protein from MHC class II alpha and beta chains in the presence of cathepsin B but not cathepsin D (11). Mutants of putative cleavage sites in the Ii protein confirm the role of residues in the R^{78}-K^{80}-K^{83}-K^{86} region (within the sequence of the Ii-Key peptide), in the final cleavage and release of the avidin-labeled Ii fragments that are still immunoprecipitated with MHC class II alpha and beta chains using anti-MHC class II antibodies (15). MHC class II protein adherence of the Ii-Key motif segment of the Ii protein (murine Ii(77-92) sequence: LRMKLPKSAKPVQMR; termed "Ii-Key"), appears to regulate lability of the antigenic peptide binding site on MHC class II molecules to accept antigenic peptides. Proteolytic cleavage in or around R^{78}-K^{80}-K^{83}-K^{86} in the Ii-Key motif of Ii protein or cleaved fragments which still adhere to MHC class II molecules, terminates the effect on binding site lability. Nevertheless, the Ii-Key allosteric site on the MHC class II molecules remains intact, even on the cell surface as judged by the activity of Ii-Key peptides on paraformaldehyde-fixed antigen presenting cells (13). However, there are few ambient peptides capable of acting at the Ii-Key allosteric site on cell surface MHC class II molecules to release antigenic peptides for two reasons. First, such sequences (hydrophobic-cationic side chains motifs) are targeted for proteases. Secondly, the motif in the Ii-Key regulatory segment of the Ii protein, XOXOX where X is a member of the group LIVFM and O is any other amino acid, is highly

Table 1. Alignment of Ii-Key peptide based on crystallographic images with Ii-CLIP and HA(306-318) antigenic peptide in HLA-DR4

Binding groove positions	**123456789**
	M^{91}
Human Ii CLIP(Ii81-99)	LPKPPKPVSK MRMATPLLM
Human Ii-Key (Ii77-92)	LRMKLPKPPKPVSK MR
Murine Ii CLIP(Ii81-98)	LPKPPKPVSK MRMATPLLM
Murine Ii-Key (Ii77-92)	LRMKLPKSAKPVSQ MR
Core Ii-Key (77-83)	LRMKLPK
Influenza HA (306-318)	PK YVKQNTLKLAT

The linearly aligned relationships among Ii-Key, CLIP and MHC class II epitope (HA306-318) sequences, relative to MHC class II binding groove, as deduced from X-ray crystallographic images (19).

suppressed ($P < 0.005$) in proteins in general (17). That is, the Ii-Key regulatory motif evolved from a sequence which is very rarely found in natural proteins. That is, the chance that a sequence would act on cell surface MHC class II molecules to release bound antigenic peptides is very low. (Table 1).

In studying the role of fragments of the Ii protein generated during the staged cleavage and release of Ii during antigenic peptide binding to MHC class II molecules, we synthesized the murine Ii-Key peptide (18). This peptide was synthesized because it contains six positively charged side chains, no negatively charged side chains, many alternating hydrophobic residues, and four prolines. This cluster of residue suggested a tightly kinked, positively charged knob which might attract a protease or "exchangease" to the MHC class II alpha,beta,Ii trimer. Antibodies to the peptide recognized the peptide and denatured Ii protein, but did not recognize the Ii protein in nonionic detergent-solubilized trimers.

Subsequently, this peptide was found to enhance greatly *in vitro* presentation of I-E-restricted antigenic peptides to responding T hybridomas (1). This "Ii-Key" peptide enhanced *in vitro* presentation of antigenic peptides by living or paraformaldehyde-fixed antigen presenting cells to murine T-cell hybridomas (20). Nested C-terminal deletions of Ii-Key showed maximal activity in the N-terminal Ac-LRMKLPK-NH_2, with half-maximal activity being retained with Ac-LRMK-NH_2. Structure-activity relationship studies of 160 homologs revealed many homologs with significantly greater activity than the original 15-amino acid peptide (21). The mechanism was explored further by assaying binding or release of biotinylated hMBP(90-102) from purified exomembranal human MHC class II molecules. With purified soluble exomembranal HLA-DR1 protein, the existence of an allosteric site was indicated by competitive binding experiments with biotinylated Ii-Key peptides or antigenic peptides (22). These experiments support the model that acting through an allosteric site on HLA-DR1 molecules, Ii-Key regulates the binding of a second, epitope-only hMBP(90-102) peptide to HLA-DR1 molecules (Table 2).

The potency of presentation of an antigenic epitope from pigeon cytochrome C, PGCC(95-104), was enhanced about 200 times *in vitro* when the N-terminus of the antigenic peptide was linked covalently through a simple chemical bridge to the C-terminus of the Ii-Key peptide, forming an Ii-Key/antigenic epitope hybrid (14). In mouse immunizations, the Ii-Key/HIV Gag(46-59) hybrid significantly enhanced the potency of the Gag epitope as ELISPOT-measured T-cell IFN-gamma responses (23). In addition, an Ii-Key/HER-2/neu MHC class II epitope peptide induced much greater IFN-gamma release fromperipheral blood mononuclear cells of breast cancer patients than did the comparable HER-2/neu MHC class II epitope-only peptide (24) (Table 3, Figure 1).

3.2. Hypothesis on the mechanism of action of Ii-Key/MHC class II epitope hybrid peptides

Our experiments support the view that Ii-Key peptides act at an allosteric site on MHC class II molecules to facilitate charging of vaccine peptides into the antigenic peptide binding site and their presentation. The allosteric site appears to be located just a few amino acids away from the end of the groove holding the N-terminus of the antigenic peptide. Binding of a ligand to the allosteric site induces a conformational change in the antigenic peptide-binding groove such that it adopts a more accessible conformation. The allosteric-site ligand, with a lesser binding affinity than the antigenic peptide for the antigenic peptide-binding groove (25), dissociates to allow stabilization of the MHC class II/epitope complex ("the clamshell closes"). Our results are consistent with those of others. Hammerling's group (26) has demonstrated that the N-terminal segment of CLIP (81-91) loosens the binding groove and releases the core CLIP (81-105) and other self epitopes from the groove. Without CLIP (81-91), the core CLIP (91-105) cannot be released from the groove even at acidic conditions. Their results indicate that CLIP (81-91) may bind at a site, which is adjacent to the N-terminus of the binding groove. They have also shown that core CLIP (91-105) binds to the groove and competes with antigenic peptides. This was also predicted by Rammensee *et al.*from a complete list of MHC class I and class II epitopes known at the time (27). X-ray crystallographic images of the Ii-derived peptide CLIP in the antigenic peptide binding groove of MHC class II molecules, indicates that methionine^{91} (M^{91}) binds to the P1 site of the groove (21). If Ii-Key, Ii protein and CLIP all maintain their registry with respect to the primary amino acid sequence, Ii-Key can be expected to lay outside the antigenic peptide binding groove. The relative alignments among Ii-Key, CLIP and the crystallographic placement of an influenza hemagglutinin antigenic peptide (HA) (19) in the peptide binding groove of MHC class II molecules is illustrated in Figure 2.

Table 2. Activities of Ii-Key homologs

Sequence	Response	
	E^d	E^k
	1.0	1.0
YRMKLPKSAKPVSQMR	13.6	1.0
RMKLPKSAKPVSQMR	13.3	1.0
KLPKSAKPVSQMR	3.4	0.7
PKSAKPVSQMR	2.6	0.8
SAKPVSQMR	4.5	0.7
YRMKLPKSAKPVSQ	16.9	2.0
YRMKLPKSAKPV	21.7	1.0
YRMKLPKSAK	32.0	1.2
Ac-YRMKLPKSAK-NH_2	39.3	6.9
Ac-LRMKLPKSAK-NH_2	47.1	7.6
Ac-YRMKLPKSA-NH_2	39.2	7.2
Ac-YRMKLPKS-NH_2	42.8	15.3
Ac-YRMKLPK-NH_2	36.3	15.5
Ac-LRMKLPK-NH_2	39.8	15.9
Ac-YRMKLP-NH_2	19.9	18.6
Ac-YRMKL-NH_2	7.1	15.5
Ac-YRMK-NH_2	2.3	14.6
Ac-YRM-NH_2	1.0	5.6

Ac-YRMKLP-NH2 (Ed) and Ac-YRMK-NH2 (Ek) were the shortest, most active peptides in the truncation series. N- and C-terminally blocked peptides were more active than their unblocked counterparts. For these assays, MHC class II-positive APC, treated with mitomycin C, were incubated for 24 h with antigenic peptide-specific T cell hybridomas, at a submaximal dose of the respective antigenic peptide, and different concentrations of the series of Ii peptides. The concentrations of antigenic peptides used were 0.4 μmol/L of HEL(l06-116) for Ed and 0.075 μmol/L THMCC(82-103) for Ek. The presented data were generated using 64 μmol/L of each Ii peptide. Interleukin released by the T hybridoma cells was quantified by [3H]TdR incorporation by interleukin-dependent HT-2 cells, using aliquots of culture supernatant. The values presented, 'Times Baseline Response', equaled the CPM of (T + APC + Ag peptide + hybrid series peptide)/ CPM of (T + APC + epitope-only peptide). The means of triplicate wells had an average SEM of 10%. The T cell response to antigenic peptide alone ('None') was designated as the baseline value 1.0. These data led to the choice of Ii-Key "core" motifs of 4 or 7 amino acids for assay in the Ii-Key/MHC class II hybrids of Table 3. Figure and table with permission from 20,21.

Table 3. Biological activities of Ii-Key/MHC class II epitope hybrid peptides with variable spacers between the Ii-Key core motif and an antigenic epitope

	SEQUENCE			
HYBRID #	**Ii-Key**	**SPACER**	**ANTIGEN**	**SYMBOL**
	Ac-		IAYLKQATAK-NH2	X
1	Ac-LRMK-	ava*-	IAYLKQATAK-NH2	○
2	Ac-LRMK-	ava-ava-	IAYLKQATAK-NH2	□
3	Ac-LRMK-	LPKS-	IAYLKQATAK-NH2	●
4	Ac-LRMK-	LPKS-AKP-	IAYLKQATAK-NH2	■
5	Ac-LRMK-	LPKS-AKP-VSK-	IAYLKQATAK-NH2	◆

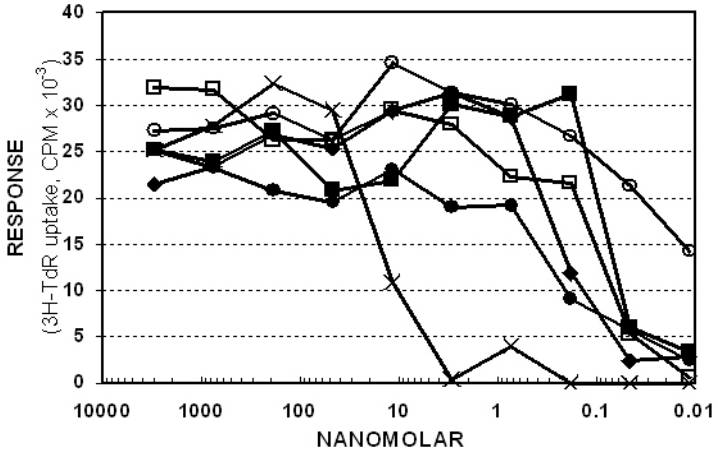

Figure 1. Potency of Ii-Key Hybrids. The immunological response to the antigenic epitope measured by tritiated thymidine uptake (y axis in thousands of counts/min), is presented as a function of the dilution factor of the hybrid (1:4 serial dilution from a 3 uM stock solution). The symbols for the respective hybrids and their structures are presented in Table 3. These results show that a simple 4-amino-acid Ii-Key motif with one of the shortest spacers has potent activity in this assay. Adapted with permission from 1.

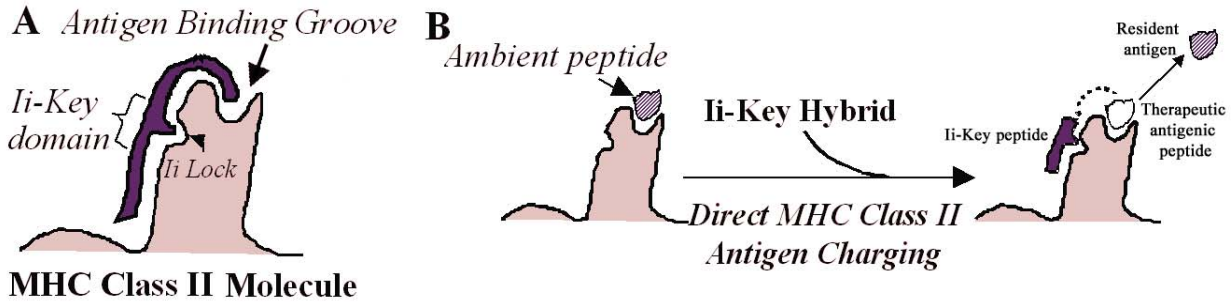

Figure 2. Direct charging of MHC class II molecules with Ii-Key hybrid vaccine. A: Ii-Key, a small domain on the Ii protein (black), interacts with Ii lock, an allosteric site distal to the antigen binding groove of MHC class II molecules. B: Ii-Key hybrids consist of an Ii-Key peptide linked to a therapeutic antigenic peptide ; when they bind to previously charged MHC class II molecules, the resident antigen ischarged and the antigenic end of the hybrid occupies the antigen binding groove.

4. DO Ii-KEY MOTIFS OCCURRING NATURALLY IN ANTIGENIC PROTEINS REGULATE SELECTION AMONG POTENTIAL MHC CLASS II EPITOPES?

The presence of Ii-Key motifs N-terminal to a potential MHC class II epitope in a protein antigen, might enhance selection of such an epitope, relative to other MHC class II epitopes without N-terminal Ii-Key motifs. In order to evaluate this possibility, we counted the frequency of Ii-Key motifs N-terminal and C-terminal to known MHC class II epitopes, as a function of spacer lengths of 3 to 6 amino acids, in a series of classical antigens. In this study the motif was defined arbitrarily to comprise a segment of 5 contiguous amino acids containing at least two amino acids of the group comprising Leu, Ile, Val, Phe, and Met, and at least one of the group comprising His, Lys, and Arg, where that contiguous 5 amino acid segment is separated by 3 to 6 amino acids from the N-terminal residue of a MHC class II-presented epitope. While a preliminary study indicated a greater frequency of such appropriately spaced motifs N-terminally to MHC class II epitopes than C-terminally, this hypothesis is subject to further experimental testing.

There are several immediate practical values to proving that naturally occurring Ii-Key motifs contribute to the selection of MHC class II epitopes in an antigen. One use is in prioritizing, and thus often reducing, the number of candidate peptides selected for synthesis and testing. Another is the ability to either reduce the immunogenicity of a therapeutic protein or to enhance the immunogenicity of an antigen. For example in the case of allergens, one might actually wish to rebalance the immune response to a Th1 phenotype by enhancing immunogenicity (28).

5. DESIGN OF Ii-KEY/MELENOMA gp100 (46-58) MHC CLASS II HYBRIDS

A principal antigen for studying immunogenicity is human gp100, which has been a good source of MHC class I and MHC class II epitopes for therapeutic vaccination trials (29-32). CD4+ lymphocytes from more than 75 % of melanoma patients recognize gp100 (33), and CD8+ tumor infiltrating T cells also recognize gp100 (34). Some gp100 peptides stimulate lymphocytes of melanoma patients who are disease-free after therapeutic intervention, but not lymphocytes from healthy donors (32). Immunization with both MHC class I- and MHC class II-restricted epitopes from the same or different melanoma-related antigens might increase the therapeutic efficacy of CTL by activating intermediary dendritic cells (35).

We focused on a DR4 transgenic mouse model to study the effect of Ii-Key/MHC class II epitope hybridization on enhancement of vaccination with h-gp100(46-58). Others had already found that immunization of HLA-DR4-IE transgenic (Tg) mice with recombinant h-gp100 protein followed by screening of candidate epitopes (identified with a computer-assisted algorithm for HLA-DRB1*0401-presented epitopes) identified h-gp100(46-58) and its clinical relevance (29). We tested a homologous series of Ii-Key/MHC class II hybrids with that epitope, varying systematically the structure and length of the spacer connecting the Ii-Key moiety and the MHC class II epitope in order to identify optimal Ii-Key/melanoma gp100(46-58) homologs for clinical trials. We found that the Ii-Key moiety enhances the potency of the gp100(48-58) MHC class II epitope *in vivo* in terms of epitope-specific CD4+ T cell activation.

5.1 Algorithm and rationale for the design of Ii-Key/MHC class II epitope hybrid vaccine peptides

Analysis of the gp100(46-58; RQLYPEWTEAQRL) peptide using two computer epitope prediction programs, (http://syfpeithi.bmiheidelberg.com/scripts/MHCServer.dll/home.html) and (http://www.imtech.res.in/raghava/propred/index.html) indicated the HLA-DR4-presented epitope to be LYPEWTEAQ (amino acid L^{48} occupies the P1 site of MHC class II molecules). A primary objective in the design of Ii-key/MHC class II epitope hybrids was to determine the effects of spacer length and requirements for natural sequence residues N-terminal to the P1 site residue of the HLA-DR4-presented epitope in gp100(46-58). Prior studies by others of MHC class II epitope peptides generally have found a requirement for non-epitope or "flanking" residues at both the N- and C-terminal of the MHC class II epitope (36). In order to evaluate the role of such additional "epitope flanking" amino acids N-terminal to the P1 residue, we synthesized hybrids extending the natural sequence at the

N-terminal end by one, two or 3 amino acids. For incorporation during peptide synthesis, the non-natural amino acid 5-aminopentanoic acid (-ava- linker) was carboxyl-activated and added to replace 2.5 amino acids of backbone length. The ava linker was actually preferred over amino acids of the Ii protein sequence as spacer due to simplicity. Similarly, series of Ii-Key/Tyr(365-381) and Ii-Key/Her(776-790) hybrids were also synthesized. Additional considerations are indicated below:

5.2. Preference for polymethylene chain versus residues of the native sequence N-terminal to the P1 site residue

Prior studies by others of MHC class II epitope peptides have generally found a requirement for 2 non-epitope or "flanking" residues at both the N- and C-terminal of the MHC class II epitope. Such residues have a "stabilizing effect," leading to an improved potency of presentation, versus peptides comprising solely a 9-amino-acid epitope. Such stabilization can be speculated to result from the following effects. a) Ionic repulsion or attractions might occur between the N-terminal peptidyl backbone ammonium or carboxylate groups with ionic side chains of neighboring MHC class II residues. Such an interaction at the C-terminus has been reported. Extension of the N- or C-termini by 2 amino acids at both ends eliminates such effects. b) Interactions of side chains and/or peptidyl backbone imido and carboxyl groups with side chains of neighboring MHC class II residues. Such interactions cannot occur in Ii-Key hybrids, for amino acids replaced by the polymethylene linker, for example where 5-aminopentanoic acid immediately precedes the P1 site residue.

5.3. Protease protection

After injection into mice, peptides are degraded by proteolytic cleavage by exopeptidases (removing one or two amino acids at a time from N- or C- termini) or by endopeptidases (cleaving potentially anywhere within a peptide, but usually at selected residues fitting a pocket of the enzyme). N-acetylation and C-amidation block exopeptidases, as done here. Endopeptidase action can be inhibited by replacing the peptidyl backbone with: a) uncleaved homologs, e.g., statins (derivatives of 3-aminohexanoic acid), b) non-natural amino acids, e.g., 3-methyl homologs of natural amino acids such as leucine, and c) a polymethylene bridge or other uncleaved spacer. Here we used -ava- to replace 2.5 amino acids of backbone length. For incorporation during peptide synthesis, the non-natural amino acid 5-aminopentanoic acid is carboxyl-activated and added, as are other natural amino acids.

5.4. Enhanced solubility

Replacement of hydrophobic side chain amino acids in a spacer segment with the polymethylene bridge of 5-aminopentanoic acid enhances solubility. Hydrophobicity is not a property of the hybrophobic residues of the Ii-Key hybrid spacer, but instead reflects the Gibbs free energy cumulatively over the wall of water molecules at their interface with hydrophobic atoms of the solute. That is, water molecules within the liquid water environment are rapidly forming and dissolving hydrogen bonds with each other, thus stabilizing those molecules in solution at a lower free energy than possessed by water molecules along a hydrophobic surface to which they cannot hydrogen bond. The larger the surface area requiring a wall of higher energy water molecules, the more water molecules are in the higher Gibbs free energy state. The methylene bridge of 5-aminopentanoic acid presents close to no hydrophobic surface; n-butanol is very soluble in water. Amino acids with a high degree of hydrophobicity include leucine (L), isoleucine (I), valine (V), phenylalanine (F), methionine (M), alanine (A), tryptophan (W), asparagine (N) - the amidated form of aspartic acid (D), and glutamine (Q) - the amidated form of glutamic acid (E). The more a peptide is composed of such amino acids, the less is its solubility. In the case of Ii-Key hybrids of gp100(46-58), hydrophobic side chains of N and Q present in gp100(45-58) have been omitted in gp100(48-58) , which was shown to have greater vaccine potency (Table 4).

5.5. Limiting autorelease of hybrids by use of shorter spacers

In our prior studies greater potency *in vitro* was associated with shorter spacers (14, 23). That fact lead to the hypothesis that the Ii-Key moiety of a hybrid first interacts with the allosteric site lying outside the antigenic peptide binding site (predicted by the overlapping alignments of the Ii-CLIP peptide and the hemagglutinin antigenic peptide in the crystallographic studies of Wiley and colleagues (19, 21). Upon replacing the endogenously bound antigenic epitope peptide with the epitope tethered to the Ii-Key moiety, the affinity of the epitope in the antigenic epitope binding trough far exceeds the affinity of the Ii-Key moiety or the allosteric site, and the Ii-Key moiety is pulled from its regulatory site, thus precluding "auto-release," i.e., catalysis of release of the antigenic epitope by action of the tethered Ii-Key moiety at the allosteric site.

6. USE OF Ii-KEY/MHC CLASS II EPITOPE HYBRIDS AS ANTI-CANCER, HELPER T CELL VACCINES

We have focused on enhancing the presentation to helper T cells of three clinically relevant MHC class II epitopes. These epitopes are gp100(46-58), Tyr(365-381), and HER-2/neu(776-790). Epitope-only peptides have been tested in clinical trials. For each of these epitopes, we have studied the effect of variable structure and length of the "spacer" element on potency of presentation of Ii-Key/MHC class II epitope hybrid vaccine peptides. The spacer comprised a simple polymethylene bridge with or without extension from the N-terminus of a putative MHC class II epitope, with amino acids of the primary sequence of the natural antigen. These data support a potential clinical utility of Ii-Key/MHC class II epitope hybrids containing these MHC class II epitopes.

6.1. Ii-Key enhances *in vivo* priming of CD4+ T cells to the gp100(46-58) epitope in DR4 transgenic mice

A homologous series of Ii-Key/MHC class II hybrids systematically varying the structure and length of the spacer connecting the Ii-Key (LRMK) segment to the gp100(46-58), DRB1*0401-restricted melanoma epitope was

Table 4. Ii-Key enhances *in vivo* priming of CD4+ T cells against the gp100(46-58) epitope

Peptide	Sequence	Response
gp100(46-58)	Ac- RQLYPEWTEAQRL-NH_2	7
gp100(45-58)	Ac-LRMK-*ava*- NRQLYPEWTEAQRL-NH_2	2
gp100(47-58)	Ac-LRMK-*ava*- RQLYPEWTEAQRL-NH_2	4
gp100(48-58)	Ac-LRMK-*ava*- LYPEWTEAQRL-NH_2	20

Splenic mononuclear cell from groups of HLA-DR0401 transgenic mice, which were vaccinated s.c. with 60 nmole of each respective peptide in an emulsion with IFA and 60 μg of CpG, were ELISPOT-assayed for IFN-γ cytokine recall response to the native gp100(46-58) epitope-only peptide. Response (mm^2) equals the product of mean spot number times the mean spot area per 10^6 cells of triplicate wells on a per mouse basis. The Ii-Key/gp100(48-58) hybrid elicited much higher frequencies of IFN-γ responders, producing larger spots, than did either the epitope–only peptide A or the other hybrids. Adapated with permission from 24.

Table 5. Ii-Key enhances *in vitro* priming of CD4+ T cells against the Tyr(365-381) epitope

Peptide	Sequence	Response
Tyr(365-381)	Ac- ALHIYMDGTMSQVQGSA-NH_2	6
Tyr(369-381)	Ac-LMRK-ava- YMDGTMSQVQGSA-NH_2	7
Tyr(370-381)	Ac-LRMK-ava- MDGTMSQVQGSA-NH_2	15

PBMC from a normal donor were incubated with either Tyr(365-382) free epitope or homologous Ii-Key hybrids. Response (mm^2) equals the product of mean spot number times the mean spot area per 10^5 cells in duplicate wells. In addition to inducing more responding cells, the Ii-Key/Tyr(370-382) hybrid generated larger spots. That effect reflects increased potency of immune response mediated by the Ii-Key moiety.

Table 6. Ii-Key enhances *in vitro* priming of CD4+ T cells against the HER(776-790) epitope

Peptide	Sequence	Response
HER-2/*neu*(776-790)	Ac- GVGSPYVSRLLGICL-NH_2	0.6
HER-2/*neu*(776-790)	Ac- LRMK- GVGSPYVSRLLGICL-NH_2	6.0
HER-2/*neu*(776-790)	Ac- LRMK-ava- GVGSPYVSRLLGICL-NH_2	1.0
HER-2/*neu*(777-790)	Ac- LRMK-ava- VGSPYVSRLLGICL-NH_2	84.0
HER-2/*neu*(778-790)	Ac- LRMK-ava- GSPYVSRLLGICL-NH_2	3.0
HER-2/*neu*(779-790)	Ac- LRMK-ava- SPYVSRLLGICL-NH_2	2.0

Total PBMC from a healthy donor were cultured with either HER(776-790) epitope-only peptide or Ii-Key/HER(776-790) hybrids. Response (mm^2) equals the product of mean spot number times the mean spot area per 10^5 cells in duplicate wells. The Ii-Key/HER(777-790) induced a much greater magnitude of immune response than did the HER (776-790) epitope-only peptide.

synthesized. The immunogenic activity of Ii-Key/gp100(46-58) hybrids was compared to the gp100(46-58) epitope-only peptide in the DRB1*0401 transgenic mice (Table 4). DR4-IE transgenic mice express the HLA-DRA-IE alpha and HLA-DRB1*0401-IE beta chimeric genes with the alpha1 and beta1 domains of human HLA-DRA and HLA-DRB1*0401 molecules, respectively. The remaining domains are from the murine I-E^d- alpha2 and I-E^d-beta2 chains, respectively (37). Each immunogen (60 nmole) was emulsified in IFA with 60 ug of synthetic phosphorothioate-modified CpG ODN 1826, 5' TCC ATGACGTTC CTG ACGTT 3' immunostimulatory nucleotide (CpG motif underlined). Three weeks after vaccination, bulk culture splenocytes (10^6/well) from individual spleens were tested for recall responses to the gp100(46-58) epitope-only peptide (5 ug/ml) in a 96-well, pre-coated IFN-γ ELISPOT plate. The results in Table 4 show that the response is enhanced upon its presentation within an Ii-Key/MHC class II epitope hybrid peptide, and that the shorter/simpler the spacer, the greater the response in this model.

6.2. Ii-Key enhances *in vitro* priming of CD4+ T cells by the Tyr(365-381) melanoma epitope in normal donor lymphocytes

A series of Ii-Key hybrids including the human MHC class II Tyr(365-381), DRB1*0401-restricted epitope of tyrosinase, a melanoma-associated antigen, were constructed. Peripheral blood mononuclear cells (PBMC) from a healthy individual were incubated with either the native Tyr(365-381) epitope-only peptide (50 ug/ml) or each of the Ii-Key/tyrosinase hybrids (50 ug/ml) (Table 5). Recombinant human IL-12 (200 pg/ml; 50 pg/10^6 cells) and IL-2 (5 U/ml) were added on days 4 and 8 of culture. The microcultures were rested in plain medium on day 12 of culture, and a week later were stimulated with irradiated, allogeneic, genotype-matched, EBV-transformed lymphoblastoid cells for 48 hr in an IFN-gamma ELISPOT plate, all wells being pulsed with the Tyr(365-381) epitope-only peptide. The Ii-Key/Tyr(370-382) hybrid with the shortest spacer (Table 5) generated larger spots than did the Tyr(370-382) epitope-only peptide, reflecting increased potency of immune response mediated by the Ii-Key moiety (Table 5).

6.3. Ii-Key enhances *in vitro* priming of CD4+ T cells to the promiscuous HER-2/neu (776-790) epitope in normal donor lymphocytes

A third series of Ii-Key hybrids included the promiscuous CD4+ T cell-stimulating, human HER-2/neu(777-790) MHC class II epitope. PBMC of a healthy individual (4 x 10^6/well) were incubated with each of the Ii-Key/HER-2/neu(776-790) hybrids or the native HER-

2/neu(776-790) epitope-only peptide, as described in section 6.2 (Table 6). These cultures were restimulated with autologous dendritic cells pulsed with the HER-2/neu(776-790) epitope-only peptide in a pre-coated IFN-gamma ELISPOT plate overnight. The Ii-Key/HER-2/neu(777-790) hybrid induced a much greater magnitude of immune response than did the HER-2/neu(776-790) epitope-only peptide (Table 6).

6.4. Conclusions from experimental studies

The increase in T cell stimulation created by adding an Ii-Key moiety to N-termini of MHC class II epitopes potentially enables new diagnostic and therapeutic uses. This anticipation was first supported by the greater sensitivity of Ii-Key/HER-2/neu hybrids in measuring *in vitro* responses of PBMC from patients with HER-2/neu-positive breast cancers (24). Such hybrid peptides could also become preferred vaccine peptides for immunizing breast cancer patients (38, 39). Enhancing helper T cell activation should create more potent responses against either endogenously generated or synthetic CTL epitopes (40-42). Likewise, Ii-Key/melanoma gp100 and Ii-Key/tyrosinase epitopes can be constructed with peptides already used clinically in vaccinating some melanoma patients (29, 30, 32). We have addressed several issues for optimal design and use of such vaccine peptides in humans through immunizations of HLA-DRbeta*0401 transgenic mice. This allele is a principal presenter of this epitope in humans (29, 30).

In these DR-transgenic mice, we compared T cell activation by homologous vaccine hybrid peptides, varying structure and length of the spacer to choose effective peptides for possible clinical trials. However, evaluating *in vitro* responses of PBMC from melanoma patients to such compounds is also desired considering the genetic heterogeneity of humans. Possibly, the increased potency of MHC class II epitope presentation created by the Ii-Key moiety in a hybrid, will allow clinically effective responses in "low responders" to the epitope-only peptide. There might also be skewing of response to a Th1 pattern with certain hybrid structures.

One finding was that the shorter the distance between the Ii-Key moiety and the MHC class II gp100 epitope, the more potent the activity. That is, including additional amino acids from the primary sequence of gp100 N-terminal to the P1 site residue is not needed in the spacer between the Ii-Key motif and the gp100 epitope. Hybrids B, C and D, containing longer spacer sequences, were all less potent than hybrid E, which had the shortest spacer sequence. These observations are consistent with our prior studies that similarly demonstrated greater potency among the Ii-Key hybrids with shorter spacers both *in vitro* and *in vivo* when examining nested deletion series of hybrids with a common MHC class II epitope (14, 23, 24).

A mechanistic interpretation for the greater potency of hybrids with shorter spacers can be proposed. Possibly shortening the spacer so that it is physically impossible for the epitope and Ii-Key to contact their respective sites on the MHC class II molecule simultaneously reduces the likelihood of auto-rejection of the epitope. Upon replacing the endogenously bound antigenic epitope peptide with the synthetic epitope tethered to the Ii-Key moiety, the affinity of the epitope in that antigenic epitope binding trough far exceeds the affinity of the Ii-Key moiety to the allosteric site. That is, binding of the synthetic epitope pulls the allosteric effector, the Ii-Key moiety, away from its site of action. There are coincidental benefits to finding best activity in a shorter spacer including: improved solubility upon deletion of intervening amino acids when they are relatively hydrophobic, removal of targets for endopeptidases (especially when target motifs are present), simplicity and lower cost in synthesis and purification.

These observations do not rule out the potential utility of using hybrids with longer spacers including additional amino acids of the natural sequence. Specifically, hybrids joining the Ii-Key moiety to a "promiscuously presented" peptide (i.e., a vaccine peptide presented by individuals with different HLA-DR alleles) might reveal multiple, closely overlapping HLA-DR-presented epitopes with different P1 site residues. Such epitopes might have P1 sites offset by only 1, 2, or 3 residues within the sequence of the antigen. The Ii-Key moiety might enhance presentation of each of those epitopes differently depending upon the specific allele present; clearly a range of spacer lengths still enhances presentation of the epitope in this series of homologs, albeit with varying potencies. Thus, we feel that a careful study of HLA-DR-genotyped patients with the entire series of homologs is important to resolve whether one Ii-Key motif is equally effective in enhancing the presentation of each of a series of slightly offset, overlapping epitopes within a "promiscuously presented" peptide. It is obvious that the HLA-DR4 transgenic mouse strain is a functional equivalent of only one patient, homozygous for the HLA-DRbeta*0401 allele.

As mentioned above, Ii-Key peptides enhance the binding of epitopes to the MHC class II antigenic peptide-binding groove. One advantage of this approach is that it allows direct charging of cell surface MHC class II molecules, bypassing the need for intracellular antigen processing. This type of charging is difficult if not impossible for free antigenic peptides because MHC class II molecules on the surface of cells have their antigen-binding groove occupied with endogenously charged antigenic peptides. Displacing these ambient peptides requires saturating concentrations of the therapeutic peptide. This is an entirely new strategy for augmenting the potency of MHC class II-restricted epitopes and results in a profound increase in T cell stimulation (100 to 200 times). It is a powerful tool to efficiently expand CD4+ T-cells ex vivo.

An important advantage of our technology is that it enhances vaccine efficacy in an antigen-specific manner; Ii-Key greatly enhances the potency of the covalently tethered MHC class II epitope, and the induced response is specific to the epitope. More recently, peptide conjugates targeting specific components of immune cells have been investigated. An example employs a T cell binding ligand coupled to a

peptide antigen (Ligand Epitope Antigen Presentation System - LEAPS) (43). However, the absolute specificity of immunostimulation using this technology has not been defined and these compounds have not yet been proven in the clinic. As the hybrid design strategy of Antigen Express targets charging of MHC class II molecules (and not T-cell receptors), antigen presentation to T-cells is ultimately more selective than the LEAPS technology and, therefore, would be expected to be more specific. Finally, rather than competing with other methods that have been used for enhancing vaccine efficacy, the Ii-Key technology can be applied in a complementary fashion, including, for example, cytokines and other adjuvants.

7. CLINICAL TRIALS WITH HER-2/NEU MHC CLASS II EPITOPE PEPTIDES

Preclinical peptide vaccine studies in animals and clinical trials have demonstrated the requirement for concordant vaccination with both MHC class II epitopes and MHC class I epitopes. By activating DC to a state of more potent action on CTL, Ii-Key hybrids stimulated T helper cells enhance CTL responses. T helper cells also provide stronger memory. Improving methods to stimulate T helper cell responses might enable curative peptide epitope-based immunotherapies.

7.1. T helper stimulation and cancer immunotherapy

Many studies in animals have well characterized the central role of CD4+ T cells in priming immune responses and facilitating 'memory' in a variety of tumor models (41, 44-47). These findings indicate that CD8+ T helper cell responses are highly dependent upon prior stimulation of CD4+ T helper cells. More specifically, dendritic cells require 'licensing' by CD4+ T cells before they can activate and expand CD8+ cytotoxic T lymphocytes (47). The importance of CD4+ T cells in tumor rejection is further illustrated by the fact that a significant fraction of CD8+ knockout mice still resist autologous tumor growth, while all CD4+ knockout animals appear incapable of resisting tumor growth (46). An explanation for this finding might lie in the fact that CD4+ cells alone are also capable of activating eosinophils and macrophages that produce both superoxide and nitric oxide within tumors, independently of CD8+ T cells. Finally, simultaneous vaccination of mice with a tumor-specific, CD8+ restricted epitope plus a tumor-specific, CD4+ restricted epitope (but not an unrelated CD4+ restricted epitope) results in strongly synergistic tumor protection (48).

The results of clinical trials similarly have begun to show the importance of CD4+ T cells in tumor immunotherapy of humans. The most persuasive data have come from adoptive transfer studies (49, 50). While exogenously stimulated CD8+ T cell clones had no impact on the course of disease in patients with malignant melanoma, a combination of CD4+ and CD8+ stimulated cells has shown a response rate of approximately 50% in 35 patients (51).

7.2. Active HER-2/neu immunotherapy: limitations of vaccinating with only MHC class I epitopes

HER-2/neu clinical trials have focused primarily on the feasibility of using antigen-specific HLA class I peptide vaccines to generate anti-tumor responses. Fisk *et al.* originally identified HER-2(369-377) 'E75" as an immunodominant HLA-A2 restricted epitope recognized by tumor-associated lymphocytes in patients with ovarian cancer (52). Several other types of tumors were later shown to generate immune responsiveness to HER-2/neu (369-377) (53-56). Even though administration of HER-2/neu(369-377) in IFA or GM-CSF resulted in the induction of peptide specific CTL responses in the majority of the immunized patients, significant clinical responses were not described (57-61). Similarly, while vaccinations with DC pulsed with tumor antigens induced peptide specific CTL *in vivo*, which lasted more than six months and lysed autologous tumor cell targets, significant clinical efficacy was lacking (62). HLA-A2-restricted, cytotoxic T cell lines generated by pulsing DC with three different HER-2/neu-derived peptides, HER-2/neu(369-377), HER-2/neu(435-443), and HER-2/neu(689-697), lysed primary HER-2/neu-expressing HLA-A2(+) renal carcinoma cells (56). The CTL that result from peptide vaccinations have generally demonstrated *in vitro* effector function against peptide-pulsed targets, tumor lines, and primary tumor cells (35, 55, 60). In spite of this immunological responsiveness, MHC class I vaccines have been associated with lack of a long term cytotoxic response, resulting in poor clinical outcomes (59, 63). Low level, short-lived CTL responses can be attributed to the lack of CD4+ helper T cell stimulation. Because of this deficiency, there has been particular interest in using peptides containing both experimentally determined MHC class II epitopes, and algorithm-predicted MHC class I-restricted epitopes (64). Vaccination of breast cancer patients with a mixture of three T helper epitopes, HER-2/neu (369-384), HER-2/neu(688-703), and HER-2/neu(971-984), which also encompassed HLA-A2 binding motifs, enhanced CTL precursor frequencies and provided long lasting anti-tumor responses detectable for more than one year after final vaccination (65). Also intriguing is the finding that patients demonstrated responses to additional MHC class II epitopes of the HER-2/neu protein not present in the immunizing peptides (38). This observation could be a crucial first step in breaking tolerance to HER-2/neu, as it points to potential spreading of immunological responsiveness to cryptic or subdominant epitopes within HER-2/neu, to which tolerizing clones of CD4+ T cells had never developed.

7.3. Active peptide immunotherapy for melanoma

The majority of clinical experience in the field of active peptide immunotherapy has been obtained from patients with melanoma using peptides designed to stimulate a CTL response. The data from those studies have been reviewed extensively (50, 66, 67) and will not be considered here again. While observations of immunological responsiveness are common, a review of over 400 patients indicated that objective clinical response rates were as low as 2.6% (50). A leading hypothesis for the failure to demonstrate therapeutic efficacy is the lack of CD4+ T cell activation. While a few recent trials have been performed using a combination of MHC class I and class II epitopes derived from tumor-associated antigens (gp100 and NY-ESO1), neither a clinical nor even an

immunological response to the class II peptides could be demonstrated. A possible explanation for the lack of activity of these peptides is the inherently lower affinity of epitopes for MHC class II molecules relative to the affinity of epitopes to MHC class I molecules. As we have shown above, addition of an Ii-Key moiety to a class II epitope greatly enhances the antigen-specific stimulation of CD4+ T cells both *in vitro* and *in vivo*. For this reason, we feel that Ii-Key modified class II epitopes are prime candidates for use in clinical trials.

8. SUMMARY AND PERSPECTIVE

While our focus here has been on the use of Ii-Key/MHC class II epitope hybrids as therapeutic vaccines for cancer, there is a wider range of applications both in cancer and for additional diseases. Such hybrids can be used to expand T cells *ex vivo* against a cancer-related MHC class II epitope or antigen, or for dendritic cell charging of the hybrid peptides for more effective *in vivo* immunization (68, 69). Such hybrids can boost responses that result from charging tumor-associated antigens to autologous DC for reinfusion (70, 71). The hybrids also can be used to expand T helper cells to one epitope prior to a DNA vaccination with the gene coding for the antigen containing the epitope of the hybrid (72). Such T helper enhancement should provide for a better response to the subsequent DNA vaccine; the response should be both more robust and characterized by more extensive epitope spreading to both MHC class I and class II epitopes of the encoded antigen. In fact, the level of epitope spreading might lessen the need for use of a selected set of 4-6 MHC class II epitopes, potentially covering 95% of humans with one peptide formulation. Epitope spreading is the phenomenon in which vaccination with one MHC class II epitope, followed by a protein antigen (here an endogenous tumor antigen) leads to increase T helper cell response to other MHC class II epitopes of the antigen. Epitope spreading is presumably mediated by cytokines increased by response to the vaccine epitope. When DNA vaccines are limited by an anti-vector immune response, such prior T cell expansion might be particularly useful in obtaining maximal response to a few doses of DNA vaccine. Finally, such hybrids can boost responses to antigens presented by charging or fusing tumor antigenic material to autologous DC. Comparable uses for infectious diseases are obvious. Also, with an appropriate Th2 suppressing adjuvant or dosing regimen, these compounds might be used to regulate autoimmunity. In summary, although the experiments of this report offer a specific step toward enabling better immunotherapies for melanoma, the principles demonstrated here are applicable to many forms of active immunotherapy targeting devastating diseases. Enough experience has been gained to show that better immunotherapies, involving antigen-specific CD4+ T helper stimulation, are possible (50). We show here a practical and clinically relevant means of accomplishing this.

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