Enhanced CD4+ T-Cell Response in DR4-Transgenic Mice to a Hybrid Peptide Linking the Ii-Key Segment of the Invariant Chain to the Melanoma gp100(48-58) MHC Class II Epitope

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Summary: Linking the Ii-Key functional group LRMK, through a simple polymethylene linker, to the melanoma gp100(48-58) MHC class II epitope significantly enhances the vaccine response to that epitope in DR4-IE transgenic mice. A homologous series of Ii-Key/gp100(46-58) hybrids was synthesized to test the influence of spacer length (between Ii-Key and the gp100(48-58) epitope) on in vivo enhancement of gp100(48-58)-specific $CD4^+$ T-lymphocyte responses. As measured by IFN-γ and IL-4 ELISPOT cytokine assays, the most effective vaccine hybrid was the one with a shorter linker between Ii-Key and the epitope. Mechanistic reasons for this observation are considered. This structure–activity relationship was seen with bulk and $CD4^+$ purified T cells, and both primary and secondary in vitro restimulation assays. CFA augmented the IFN-γ response and to a lesser extent the IL-4 response. CpG enhanced a strong IFN-γ response, with a negligible IL-4 response. The 3- to 5-times enhancement of the total ELISPOT responses (number of spots × mean spot area) observed after vaccination with peptides consisting of an MHC class II epitope engineered into an Ii-Key hybrid indicates a potent vaccine effect. Such constructs can be applied to many diagnostic and therapeutic uses.

Key Words: Ii-Key, gp100, vaccine, MHC class II, melanoma, T-helper cell

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A novel mechanism for boosting responses to MHC class II epitope vaccine peptides exploits a regulatory allosteric site on MHC class II molecules, which governs the tightness of binding of MHC class II epitope peptides. The normal process of MHC class II antigen charging and presentation is highly controlled to ensure 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 otherwise destined for binding to MHC class I molecules. Only after the MHC class II molecule complexes are transported into a post-Golgi compartment is the Ii protein digested away, allowing access to charging MHC class II molecules with exogenous peptides.[1–4] To study the process of concerted cleavage and release of the Ii protein and antigenic peptide charging, we assayed several synthetic Ii peptides for biologic activity.

We found that a peptide of the Ii protein, Ii(77-92; LRMKLPKPPKPVSQMR), enhanced in vitro presentation of antigenic peptides by living or paraformaldehyde-fixed antigen-presenting cells to murine T-cell hybridomas.[5] Structure–activity relationship studies of 160 homologs revealed a core sequence (Leu-Arg-Met-Lys-Leu-Pro-Lys; LRMKLPK) with significantly greater activity than the original 16-amino acid peptide.[6,7] The shortest sequence with half-maximal activity of the most potent peptide LRMKLPK contains only four amino acids (LRMK). These Ii-Key peptides appear to act at an allosteric site on MHC class II molecules to facilitate charging and presentation of vaccine peptides into the antigenic peptide-binding site.

The potency of presentation of an antigenic epitope from pigeon cytochrome C was enhanced more than 250 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.[8] 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-γ responses.[9] In addition, an Ii-Key/HER-2/*neu* MHC class II epitope peptide induced much greater IFN-γ release from peripheral blood mononuclear cells of breast cancer patients than did the comparable HER-2/*neu* MHC class II epitope-only peptide.[10] Such Ii-Key/MHC class II antigenic epitope hybrids have potential applications in vitro as diagnostics and for monitoring patient immunotherapies. As vaccines, they can be applied to control cancer and infectious diseases.[11] Here we have evaluated the vaccine potential of a clinically relevant melanoma gp100 MHC class II epitope in immunizing HLA-DR4 transgenic mice.

The design of Ii-Key/gp100(46-58) MHC class II hybrids followed the following principles and methods. Analysis of the gp100(46-58; RQLYPEWTEAQRL) peptide using two

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computer epitope prediction programs, (http://syfpeithi.bmiheidelberg.com/scripts/MHCServer.dll/home.html) and (http://www.imtech.res.in/raghava/propred/index.html), indicated that the HLA-DR4-presented epitope was LYPEWTEAQ (amino acid L-48 occupies the P1 site of MHC class II molecules). Given this putative epitope, 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-terminals of the MHC class II epitope.[12] 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 three amino acids. A polymethylene chain spacer was preferred over residues of the native gp100 sequence N-terminal to the P1 site residue of the epitope because such a simple chemical structure would interact negligibly with side chains and/or the peptidyl backbone of neighboring MHC class II residues. 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. Protection against proteases was obtained by N-acetylation and C-amidation to block exopeptidases, and by replacing the peptidyl backbone with the polymethylene linker that is not internally cleaved by endopeptidases. Solubility was incidentally enhanced by replacing spacer amino acids with the polymethylene bridge, for which the low surface area is less hydrophobic than the side chains of many naturally occurring amino acids. "Auto-release" of the antigenic epitope by the tethered Ii-Key moiety in the same hybrid was thought to be limited by the use of shorter spacers. In our prior studies, greater potency in vitro was associated with shorter spacers.[8,9] This observation 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. After 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. Use of short spacers might thus preclude "auto-release" (ie, catalysis of release of the antigenic epitope by action of the tethered Ii-Key moiety at the allosteric site).

There is clear value in using Ii-Key/MHC class II epitope hybrids to enhance both T-helper and cytotoxic T-cell responses to a cancer antigen. An optimal MHC class I-directed CTL response requires co-stimulating MHC class II-directed T-helper cell responses.[13] A review of responses by a large series of 400 melanoma patients receiving various types of peptide-, DNA- and cell-based immunotherapies found that although a few significant therapeutic responses occurred, a need for better approaches remains.[14] A principal need is generating $CD4^+$ cells that recognize MHC class II-restricted, tumor-associated antigens. $CD4^+$ T-helper cells are critical to inducing and maintaining CTL activity to tumor cells.[15,16] Further, in adoptive transfer therapy for melanoma, a 50% response rate required the addition of $CD4^+$ antitumor cells to $CD8^+$ CTL antitumor preparations.[17]

Human gp100, a well-defined target for $CD4^+$ melanoma-reactive T cells, has been an excellent source of MHC class I and MHC class II epitopes for therapeutic vaccination trials.[18–21] Lymphocytes from more than 75% of melanoma patients recognize gp100,[22] and $CD8^+$ T cells from tumor-infiltrating lymphocyte populations can also recognize gp100.[23] That peptide stimulates lymphocytes of melanoma patients who are disease-free after therapeutic intervention, but not lymphocytes from healthy donors.[21] Co-immunization with both MHC class I- and MHC class II-restricted epitopes from the same or different melanoma-related antigens is expected to increase responses and the therapeutic efficacy of CTL by activating a common intermediary APC.[24]

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) led to identification of h-gp100(46-58) and the study of its clinical relevance.[18] Now we examine a homologous series of Ii-Key/MHC class II hybrids of that peptide, varying systematically the structure and length of the spacer connecting the Ii-Key moiety and the MHC class II epitope to identify optimal Ii-Key/melanoma gp100(46-58) homologs for clinical trials. Our results indicate that the Ii-Key functional group significantly enhances the potency of the gp100(48-58) MHC class II epitope in vivo in terms of epitope-specific $CD4^+$ T-cell activation. These studies provide justification for further development of this novel agent as a vaccine for clinical trials in melanoma.

METHODS

Animals

DR4-IE Tg mice express HLA-DRA-IE alpha and HLA-DRB1*0401-IE beta chimeric genes consisting of the $\alpha 1$ and $\beta 1$ binding domains from the human HLA-DRA and HLA-DRB1* 0401-IE molecules, respectively, and the remaining domains from the murine I-E^d-$\alpha 2$ and I-E^d-$\beta 2$ chains, respectively.[25] Female DR4-IE Tg mice from Taconic Laboratories were studied in the University of Massachusetts Animal Facility, under an approved protocol.

Immunizations

DR4-IE Tg mice were immunized subcutaneously at the base of the tail with 40 nmol or 60 nmol of Ii-Key/gp100 MHC class II hybrid or epitope-only peptides, dissolved in saline and emulsified with an equal volume of either incomplete Freund's adjuvant (IFA) or complete Freund's adjuvant (CFA). In some experiments with IFA, 60 μg of the synthetic phosphorothioate-modified CpG ODN 1826, 5′ TCC ATGACGTTC CTG ACGTT 3′ immunostimulatory nucleotide (CpG motif underlined; from Oligos Etc, Wilsonville, OR) was added before emulsifying. Three weeks following the first immunization, splenic lymphocytes, or purified $CD4^+$ T cells therefrom, were ELISPOT-assayed for IFN-γ, IL-4, and IL-2 responses. An alternative immunization schedule was one booster

immunization at 2 weeks, followed at 4 weeks by the immune response assays.

Peptides

The MHC class II-restricted human gp100(46-58) epitope was originally identified in DR4-IE Tg mice by Toloukian et al.[18] Ii-Key/gp100 hybrids systematically deleting from the N-terminus of the epitope-containing segment, and also HER-2/*neu*(776-790; GVGSPYVSRLLGICL) control peptide, were synthesized to 99% purity as assessed by HPLC and mass spectrometry (Commonwealth Biotechnologies, Richmond, VA) (Table 1). The N-terminus of the gp100(46-58) epitope was linked to the Ii-Key segment (LRMK) by the flexible polymethylene spacer *ava* (5-aminovaleric acid = 5-aminopentanoic acid) spacer. All peptides were N-acetylated and C-amidated to inhibit exopeptidases. The peptides were dissolved in sterile water (2 nmol/μL) and stored at −80°C.

Purification of CD4+ T Cells

To assess gp100(46-58) specific T-helper activities, mononuclear cell splenocyte suspensions were prepared from individual mice after erythrocyte lysis with Pharmlyse (BD Pharmingen, San Diego, CA). A mixture of 100×10^6 cells (20×10^6 cells per mouse with five mice per group) were cultured with 25 μg/mL of gp100(46-58) epitope-only peptide. After 5 days of secondary culture stimulation, purified $CD4^+$ T cells were isolated with MACS CD4 (L3T4) microbeads (Miltenyi Biotec, Auburn, CA). Nonadherent cells were collected from the bulk cultures, washed, and labeled with L3T4 microbeads (10 μL of beads per 10×10^6 cells) in 90 μL of 0.01 M sodium phosphate-buffered, pH 7.4, 0.14 M NaCl solution (PBS) containing 2 mM EDTA and 0.5% BSA (PBS-EDTA). The cells were well mixed and incubated for 15 minutes in the dark at 6° to 12°C. Fluorochrome-conjugated CD4 antibody was added and incubated for an additional 5 to 10 minutes. The cells were washed by adding 1 mL PBS-EDTA and centrifuged for 10 minutes at 300*g* at 4°C. The cell pellet was resuspended in 0.5 mL PBS-EDTA per 10^8 cells for magnetic separation. Columns were placed in the magnetic field of the MACS separator and washed with 500 μL PBS-EDTA. The cell suspension was applied to the column and the effluent was collected as the negative fraction. The column was rinsed three times with 0.5 mL PBS-EDTA and placed away from the separator. Fresh PBS-EDTA 1 mL was added to the column and the $CD4^+$ T-cell fraction was flushed out with a plunger. Immunomagnetically purified $CD4^+$ Th cells following a 5-day secondary culture restimulation were assayed against the gp100(46-58) epitope only peptide-A in IFN-γ ELISPOT assay overnight.

TABLE 1. Design of gp100(46-58) Hybrids

Peptide	Sequence	Abbrev.
gp100(46-58)	Ac- RQLYPEWTEAQRL -NH_2	A
gp100(45-58)	Ac-LRMK- *ava*- NRQLYPEWTEAQRL -NH_2	B
gp100(46-58)	Ac-LRMK- *ava*- RQLYPEWTEAQRL -NH_2	C
gp100(47-58)	Ac-LRMK- *ava*- QLYPEWTEAQRL -NH_2	D
gp100(48-58)	Ac-LRMK- *ava*- LYPEWTEAQRL -NH_2	E

The Ii-Key segment of the Ii protein was linked to different amino acid of the N-terminus of gp100(46-58) one δ-aminovaleric acid residue (*ava* = 5-aminopentanoic acid) spacer.

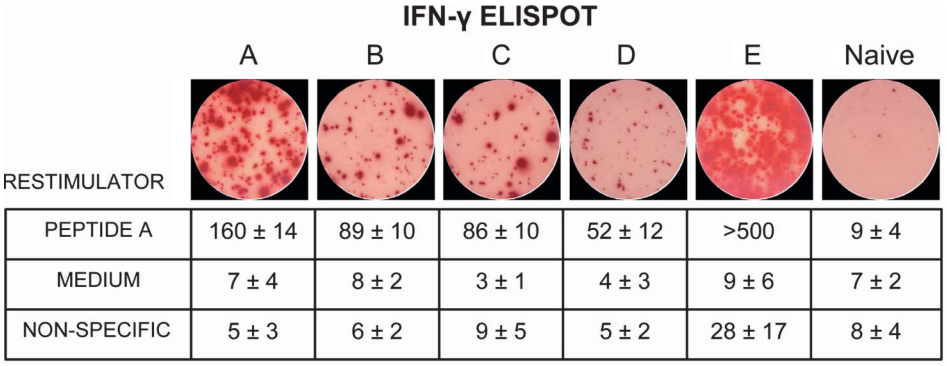
ELISPOT Assays

Both unseparated and separated $CD4^+$ T-lymphocytes were used in ELISPOT assays. Bulk culture lymphocytes (10^6 cells/well) obtained from individual spleens of immunized animals in each group were stimulated with 5 μg gp100(46-58) epitope-only peptide in 96-well immunospot 200 plates for 36 hours (Fig. 1). Immunomagnetically purified $CD4^+$ T cells were also assayed. ELISPOT assays were performed with BD Pharmingen sets for IFN-γ (cat. no. 551849), IL-4 (cat. no. 551017), and IL-2 (cat. no. 551282) according to the manufacturer's instructions. Briefly, plates were coated overnight at 4°C with the cytokine capture antibodies. The plates were blocked with 10% fetal bovine serum (FBS) in RPMI-1640 buffer for 2 hours at room temperature and washed four times with PBS containing 0.05% Tween-20 (wash buffer). Suspensions of bulk and purified $CD4^+$ T cells were added to the cytokine antibody precoated plates (Table 3). For determination of a primary response, bulk cultures were incubated directly in the plates for 36 hours (see Fig. 1); in vitro restimulation following a 5-day incubation with peptide in culture was assessed by overnight incubation of purified $CD4^+$ T cells in cytokine antibody precoated plates (Table 3). The plates were then washed five times with wash buffer. Biotinylated cytokine detection antibodies (2 μg/mL) were added for 2 hours at room temperature. The plates were washed four times with wash buffer and avidin horseradish peroxidase-conjugated (avidin-HRP) was added at 1:100 dilution from the commercial stock for a 1-hour incubation at room temperature. Avidin-HRP was removed by washing four times with wash buffer and two times with PBS. The spots were developed with HRP-3-amino-9-ethylcarbozole substrate (BD Pharmingen) for 30 minutes at room temperature. The plates were washed twice with sterile water and dried for 1 to 2 hours at room temperature. Digitalized images of spots were analyzed with a Series 1 Immunospot Analyzer and Immunospot 1.7e software (Cellular Technology Ltd, Cleveland, OH). Criteria for spot size, circularity, and color density were determined by comparing control and experimental wells.[26,27] Partially overlapping spots were separated, and also noise signal caused by substrate precipitation and nonspecific antibody binding was eliminated. Only areas meeting the specified criteria were counted as spots. Mean and standard deviations were calculated for all assays. Total spot size area was the product of mean spot area times the number of cells, per well.

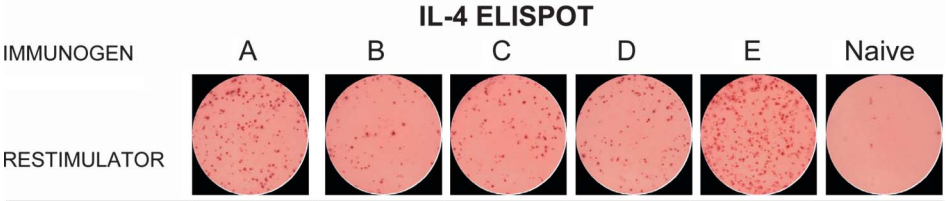
RESULTS

Ii-Key Enhances In Vivo Priming of CD4+ T Cells Against the gp100(46-58) Epitope

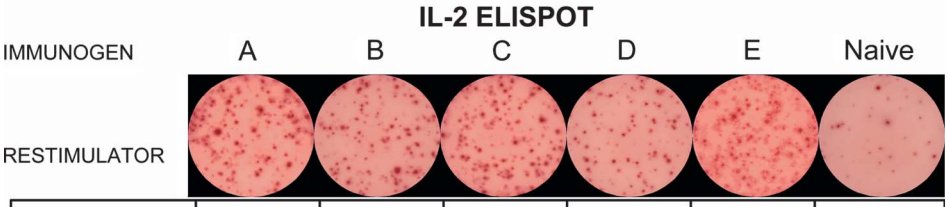
Splenic mononuclear cells from HLA-DR4-IE Tg mice, immunized with each of the peptides of Table 1 in CFA, were



PEPTIDE A	160 ± 14	89 ± 10	86 ± 10	52 ± 12	>500	9 ± 4
MEDIUM	7 ± 4	8 ± 2	3 ± 1	4 ± 3	9 ± 6	7 ± 2
NON-SPECIFIC	5 ± 3	6 ± 2	9 ± 5	5 ± 2	28 ± 17	8 ± 4



PEPTIDE A	183 ± 26	69 ± 10	96 ± 10	89 ± 27	327 ± 26	6 ± 2
MEDIUM	6 ± 3	5 ± 3	5 ± 1	16 ± 10	10 ± 56	3 ± 1
NON-SPECIFIC	11 ± 3	15 ± 6	11 ± 4	11 ± 4	16 ± 6	6 ± 4



PEPTIDE A	130 ± 11	104 ± 10	117 ± 15	73 ± 12	150 ± 10	23 ± 4
MEDIUM	18 ± 5	20 ± 4	13 ± 5	23 ± 7	33 ± 14	25 ± 13
NON-SPECIFIC	13 ± 2	15 ± 6	18 ± 8	16 ± 5	30 ± 8	20 ± 9

FIGURE 1. IFN-γ and IL-4 responses of bulk splenocytes from mice immunized with gp100(46-58) hybrids and epitope-only peptide in CFA. Five mice per group were vaccinated subcutaneously with 60 nmol of peptides in an emulsion with CFA. IFN-γ and IL-4 cytokine recall responses against the gp100(46-58) epitope-only Peptide A were directly ex vivo ELISPOT-assayed (36 hours) on pooled splenocytes. The mean number of spots and standard deviations for six replicate wells are given. Representative images of respective wells are shown. Stimulation with a nonspecific HLA-DR4-recognized HER-2/*neu* (776-790) epitope generated a negligible immune response.

ELISPOT-assayed for IFN-γ, IL-4, and IL-2. Three weeks following immunization, bulk splenic cells (primary stimulation, Fig. 1) or purified $CD4^+$ T-cells were assayed after a secondary in vitro stimulation with epitope-only peptide-A (Table 3). The Ii-Key/gp100(48-58) hybrid E (LRMK-*ava*-linked to the peptidyl amino group of the N-terminal residue of the epitope-containing segment) elicited much higher frequencies of IFN-γ and IL-4 responding cells than did either the gp100(46-58) epitope-only peptide A or other hybrids, both in the primary and secondary stimulation assays. Specificity of the gp100(46-58) immune response was confirmed by performing in vitro stimulations with an unrelated HLA-DR4-recognized HER-2/*neu*(776-790) epitope. This peptide yielded a background level of less than 10 spots/well. Gp100(46-58)-specific IL-2 secretion was not significantly enhanced with any of the hybrids (see Fig. 1). Thus, in this system, the Ii-Key motif enhances only the primary activation of epitope-specific $CD4^+$ T cells, but not apparently $CD4^+$ T cell-mediated autocrine maintenance through IL-2 production. The relative IFN-γ and IL-4 cytokine production of bulk splenic cells in the primary stimulation (see Fig. 1) was estimated by the total spot area (mean spot number × mean spot size, Table 2). Overall, the enhancement of the total ELISPOT responses observed after vaccination with peptides consisting of a MHC class II epitope engineered into an Ii-Key hybrid indicates a potent vaccine effect.

Through repeated experiments, testing cumulatively 20 to 25 mice per peptide, hybrid E was consistently the most potent, while hybrids B and C had activities comparable to epitope-only peptide A, and hybrid D was less active than any of the others. These data indicate that the Ii-Key enhancement of epitope-specific $CD4^+$ T cells relates to the length and composition of the spacer. This pattern of results was consistent whether the mice were assayed 3 weeks after a priming injection, or 2 weeks after one booster injection, which was given 2 weeks after the priming injection (data not shown).

TABLE 2. Relative IFN-γ and IL-4 Production Depicted by Total Spot Area (mm^2)

	Immunogens				
	A	B	C	D	E
IFN-γ	42 ± 4	8 ± 2	9 ± 2	3 ± 0.7	~137
IL-4	5 ± 0.8	2 ± 0.4	1.4 ± 1	3 ± 1	13 ± 1.3

Relative IFN-γ and IL-4 production of bulk splenocytes was estimated by total spot areas. Total spot area (mm^2) equals the product of mean spot number times the mean spot area. Groups of 5 mice were immunized with gp100(46-58) hybrids and epitope-only peptide in CFA.

IFN-γ Secretion Induced by Ii-Key/gp100(46-58) Hybrids or the gp100(46-58) Epitope-Only Peptide Comes From CD4+T Helper Cells

Since IFN-γ can be produced either by $CD4^+$ T cells or by macrophages in bulk splenocyte cultures, immunomagnetically purified $CD4^+$ T-cells were assayed (Table 3). The IFN-γ secreting pattern of such purified $CD4^+$ T cells was similar to that of bulk splenic cells (see Fig. 1). These results indicate that Ii-Key/gp100 hybrid E primes gp100(48-58)-specific $CD4^+$ T cells more potently in vivo than does the epitope-only peptide A.

CpG Is a TH1 Directing Adjuvant

In part toward finding a clinically acceptable adjuvant, we used CpG in the animal immunizations with IFA. An additional benefit of using CpG is the fact that it elicits a strong TH1-oriented response through recognition of its metabolites by TLR7.[28] Groups of 5 HLA-DR4-IE Tg mice were immunized with each peptide indicated in Table 1, emulsified in IFA with 60 μg CpG. The IFN-γ ELISPOT assay showed a similar pattern of Ii-Key enhancement (Table 4) compared with vaccinations with CFA (Fig. 1). Vaccinations with CFA elicited a greater immune response than those performed in CpG. However, these adjuvants differed in their induction of IL-4. Epitope-specific IL-4 secretion was enhanced by the Ii-Key hybrid using CFA (see Fig. 1) but was negligible when CpG in IFA was the adjuvant (Table 4). Even though CpG has been reported as a TH1 adjuvant downregulating TH2 responses, negligible IL-4 production might be below the ELISPOT detection limits, not necessarily reflecting the in vivo activity of such vaccinations.

TABLE 3. IFN-γ Responses of Purified CD4+ T Lymphocytes from Mice Immunized with gp100(46-58) Hybrids and Epitope-Only Peptide in CFA

	Immunogens					
Stimulator	A	B	C	D	E	Naive
Peptide A	491 ± 5	165 ± 11	110 ± 24	25 ± 5	1,182 ± 82	4 ± 2
Medium	0	2 ± 2	0	0	2 ± 2	0

Subcutaneous injection of immunogen (60 nmol) in CFA at the base of the tail. Three weeks following the immunization, splenocytes were pooled from five mice per group and restimulated in vitro with peptide A (5 μg/10^6 cells) and also a nonspecific control HER-2/neu(776-790) epitope for 5 days. Following the in vitro restimulation, cells from triplicate wells were pooled together, washed, and counted for CD4+ T-lymphocyte purification using magnetic beads. Immunomagnetically purified splenic CD4+ Th cells were assayed for IFN-γ secretion overnight. Stimulation with a nonspecific HLA-DR4-recognized HER-2/*neu* (776-790) epitope generated three to five spots. Data represent mean spot number and SD per triplicate wells.

TABLE 4. IFN-γ and IL-4 Responses of Bulk Splenocytes from Mice Immunized with gp100(46-58) Hybrids and Epitope-Only Peptide with CpG in IFA

	IFN-γ ELISPOT Responses				
	Immunogen				
Stimulator	A	B	C	E	Naive
Peptide A	75 ± 21	35 ± 22	64 ± 40	166 ± 130	8 ± 2
Medium	5 ± 5	4 ± 3	5 ± 3	5 ± 4	3 ± 1
	IL-4 ELISPOT Responses				
Peptide A	17 ± 10	13 ± 10	9 ± 3	17 ± 9	8 ± 6
Medium	4 ± 3	6 ± 3	6 ± 3	2 ± 1	7 ± 5

Five to eight mice per group were vaccinated subcutaneously with 60 nmol of peptides in an emulsion with IFA and 60 μg CpG. IFN-γ and IL-4 cytokine recall responses against the gp100(46-58) epitope-only Peptide A were ELISPOT-assayed on a per mouse basis. The mean number of spots and standard deviations for triplicate wells are given.

DISCUSSION

Insertion of the gp100(48-56) MHC class II-restricted epitope into an Ii-Key/MHC class II epitope hybrid peptide creates a novel vaccine peptide that elicits more potent $CD4^+$ T-cell responses than does the corresponding epitope-only peptide. Several issues in the optimal design and use of such a vaccine peptide for humans have been addressed through immunizations of a homologous and structurally varied set of homologs into HLA-DRβ*0401 transgenic mice. This allele is a principal presenter of this epitope in humans.[18,19]

Studies in transgenic mice are an important component in preclinical vaccine evaluation. Through these studies we seek to identify an effective vaccine peptide (among homologs) for a clinical trial and to explore an effective vaccination protocol and adjuvant. However, evaluating in vitro responses of 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 are also suggestions of skewing of response to a TH1 pattern with certain hybrid structures. In any event, the present study does answer several questions relevant to the eventual clinical use of these vaccine peptides.

First, while in this study the shorter the distance between the Ii-Key moiety and the MHC class II epitope, the more potent the activity, this is not an established rule. Our data suggest that with single epitope peptides, apparently shortest is better, but this is not a proven generalization. Our unpublished observations in a parallel and temporally more advanced study with Ii-Key/MHC class II epitopes of HER-2/neu peptides and human cells have so far indicated that when two or more overlapping, slightly offset epitopes exist within one "promiscuous

peptide," shorter is not always the best. The effect of spacer length in such promiscuous peptides upon enhancement by the Ii-Key moiety of presentation of each respective epitope has not been resolved yet.

Nevertheless, in this experimental model, including additional amino acids from the primary sequence of gp100 N-terminal to the P1 site residue in the spacer between the Ii-Key motif and the gp100 epitope was not beneficial. 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.[8–10]

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 autorejection 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. It was noted that one hybrid (D) had reduced activity. While we have not addressed all possible reasons for this observation experimentally, the decreased activity of hybrid D is consistent with the autorelease hypothesis (ie, catalysis of release of the antigenic epitope by action of the tethered Ii-Key moiety at the allosteric site). There are coincidental benefits to finding best activity in a shorter spacer, including improved solubility when intervening amino acids are relatively hydrophobic, removal of targets for endopeptidases (especially when target motifs are present), and simplicity and lower cost in synthesis and purification.

Longer hybrids might be preferred when they incorporate added amino acids of the antigenic sequence N-terminal to a region containing one or more overlapping epitopes; that is, hybrids joining the Ii-Key moiety to a "promiscuously presented" peptide (ie, 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 one, two, or three residues within the sequence of the antigen. The Ii-Key moiety might enhance presentation of each of those epitopes; 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 melanoma patients with the entire series of homologs is important. It is obvious that the HLA-DR4 transgenic mouse strain is a functional equivalent of only one patient, homozygous for the HLA-DRβ*0401 allele.

As a first step toward defining an optimal adjuvant for clinical immunization, we tested CFA (a classic adjuvant for animal studies, but not used in humans) and CpG. The mechanisms by which adjuvants stimulate innate immune responses, and thereby condition the developing antigenic epitope-specific response, triggered through T-cell receptors and antibodies, is becoming defined in detail. CFA in general leads to a TH1-type immune response, while IFA directs toward a TH2-type immune response.[29–31] CpG is also a strong TH1 adjuvant while downregulating TH2 responses.[32,33] Furthermore, heat-killed *Mycobacterium tuberculosis* in the bayol oil of FCA activates multiple Toll-like receptors (TLR), while metabolites of CpG bind to TLR7 and activate the innate immune response through the TH1 gene regulator (T-bet) signaling pathway.[28,32] We found comparable enhancement of $CD4^+$ TH1 epitope-specific activation using Ii-Key linked to an MHC class II epitope (hybrid E) using either CFA or CpG as adjuvant. Our data do show that immunization with peptides A to E together with CFA induced both IFN-γ and IL-4 (see Fig. 1), while immunization with those peptides respectively with CpG as adjuvant induced only IFN-γ (see Table 2).

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 wide range of applications both in cancer and 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.[34,35] Such hybrids can boost responses to antigens presented by charging antigenic tumor materials to autologous dendritic cells for reinfusion.[36,37] The hybrids also can be used to expand T-helper cells to one epitope prior to a recombinant protein or DNA vaccination with a gene encoding the antigen containing the epitope of the hybrid.[38] Such T-helper enhancement should provide for a better response to the subsequent DNA or recombinant protein 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 antigen. In fact, the level of epitope spreading might lessen the need for baskets of MHC class II or MHC class I epitopes, potentially covering 95% of humans with one peptide formulation. 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 dendritic cells. 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 $CD4^+$ T-helper stimulation, are possible.[39] We show here a practical and clinically relevant means of accomplishing this.

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