



HER-2 peptides p776 and F7, N-terminal-linked with Ii-Key tetramer (LRMK) help the proliferation of E75-TCR⁺ cells: The dependency of help on the side chains of LRMK-extended peptide pointed towards the T cell receptor

YUFENG LI^{1,2}, SATOSHI ISHIYAMA^{2*}, SATOKO MATSUEDA^{2*},
NAOTAKE TSUDA² and CONSTANTIN G. IOANNIDES²

Departments of ¹Melanoma and ²Gynecologic Oncology,
The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

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Abstract. The objective of this study was to determine whether peptides consisting of the Ii-Key peptide LRMK linked to the N-terminal ends of HER-2 peptides would stimulate the expansion of antigen-specific E75-TCR⁺CD8⁺ cells. The peptides tested were N-acetylated and linked to an α -amide at the C-terminus; some of the peptides contained ϵ -aminovaleric acid (Ava) between the LRMK and the HER-2 peptide. Of the seven LRMK-HER-2 peptides tested to date, three effectively induced IFN- γ production by peripheral blood mononuclear cells (PBMCs) from healthy donors and women with ductal carcinoma *in situ*. A fusion peptide, LRMK-Ava-HER-2(777-789), was more immunogenic than the natural HER-2(777-789) antigen, G89, with regard to IFN- γ production. In combination with the CD8-activating peptide E75 [HER-2(369-377)] LRMK-p776 and LRMK-Ava-F7 induced the proliferation of E75-TCR^{Med+Hi} CD8⁺ cells to a greater extent than did 1,000 or 5,000 nM of E75 alone, respectively. The induction effects were strongest at 600 nM for LRMK-p776 and 3,000 nM for LRMK-Ava-F7. At 3,000 nM, LRMK-p776 was cytotoxic to PBMCs. LRMK-

p776 and F7 had a similar specificity and preferences for binding HLA-DR molecules. The molecular modeling of HLA-DR:LRMK-p776 and HLA-DR:LRMK-Ava-F7 complexes revealed the side chains of the peptides, which pointed towards the T-cell receptor. Differences in side chain orientation introduced by various N-terminal extensions of MHC class II-bound peptides should be important for directing CD4⁺ cells to stimulate CD8⁺ cells or for eliminating regulatory T cells in cancer immunotherapy.

Introduction

We investigated the ability of seven overlapping HER-2 peptides encompassing the region HER-2(774-789) linked to LRMK to activate T cells (Murray *et al* unpublished data). The Ii-Key activator, the tetrapeptide LRMK, was connected to HER-2 peptides either directly via a peptide bond with their N-terminus or indirectly through ϵ -aminovaleric acid (Ava). Ava is an intermediary in the degradation pathway of lysine that contains an amino group at position 5. Peptides linked to LRMK through Ava have a peptide bond between the C-terminus Lys in LRMK and the δ carbon of Ava, instead of the canonical C- α 'C- α peptide bond found in most proteins. The rationale for introducing Ava by Humphreys *et al* was that the four consecutive methylene groups would form a flexible uncharged C chain that would allow the free orientation of the LRMK towards the Ia chain-binding pocket outside the peptide-binding groove (1-5).

Six of the seven HER-2 peptides tested had the same C-terminal leucine. The length of the N-terminus of HER-2 was increased by the addition of three natural amino acids, Gly-Val-Gly, to Ser⁷⁷⁷ (Table I). Ser⁷⁷⁷ is the first N-terminal amino acid of the peptide G89 [HER-2(777-789)] (6). We showed that peptide G89 activated T cells from the healthy donors and patients with breast cancer and that G89 seemed to bind to HLA-DR4 molecules with a higher affinity than did other HER-2 peptides (6).

Previous studies by us and by the Disis and Cheever laboratories identified an area in the HER-2 protein that

Correspondence to: Dr Constantin G. Ioannides, Department of Gynecologic Oncology, Box 304, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
E-mail: cioannid@mdanderson.org

*Contributed equally

Abbreviations: Ava, ϵ -aminovaleric acid; AE37, LRMK-p776 [LRMK-HER-2(774-788)]; AE47, LRMK-Ava-F7 [LRMK-Ava-HER-2(776-788)]; TCR, T cell receptor

Key words: HER2/neu, MHC class II, Ii-Key peptide, T-helper cells, cancer

contains peptides that activate T cells in humans and rodents (7-10). Although many HER-2 protein sequences have been submitted to the GenBank, a comparison of findings across studies has been difficult since the numbering of the residues and the positions of the amino acids have not been reported consistently. One of the purposes of this study was to impose some consistency in reporting. Specifically, the two peptides identified by the Ioannides laboratory are HER-2 (776-788) (F7) and HER-2(777-789) (G89); the peptide identified by the Disis and Cheever laboratories, HER-2(774-788) or p776, represents F7 with two additional amino acids at the N-terminus (7).

F7 and G89 have been shown to activate the production of IFN- γ and the proliferation of T cells from patients with ovarian or breast cancer (6-8). Recall responses to p776 *in vitro* were originally thought to require previous patient vaccination *in vivo* (7,9). The activating ability of F7 and G89 was enhanced by IL-12 (11), which was also true for the Disis-Cheever peptide 776 (12).

In another study, Ii-Key peptides linked to CD4-activating antigens from pigeon cytochrome C were found to activate secondary/recall responses from a pigeon cytochrome C-specific CD4⁺ murine hybridoma line (1-4). However, most of the Ii-Key peptides have been reported to be toxic at concentrations of >10,000 nM (2). It remains unclear whether the toxicity is due to the basic N-terminal peptide, the C-terminal α -amide, or the hydrolysis products of these peptides.

In the *in vitro* and *in vivo* models used to date, some Ii-Key-linked peptides have been stronger activators than others (1-5). Our studies with human cells revealed that only one of four distinct candidate immunopotentiating Ii-Key peptides LRMK had stronger T-cell activating effects than the free 'unprotected' peptide G89 [HER-2(777-789)] (13). This peptide sequence LRMK was used in this study linked to HER-2 peptides.

The fact that LRMK-p776 did not include Ava offered an opportunity to investigate its binding potential to human MHC class II molecules and to model its structure in complex with HLA-DR1. We found that the potential binding affinity for HLA-DRB1*0102 was similar for the natural peptide, p776, F7 and LRMK-p776. These same peptides also had a similar potential binding affinity to HLA-DRB1*0101. Given this observed similarity, we constructed models of each peptide in complex with HLA-DR1. We found differences in the activating potential and the orientation of the N-terminal side chains of LRMK-p776 and LRMK-Ava-F7.

Materials and methods

Donors. Peripheral blood mononuclear cells (PBMCs) used in this study were from women with ductal carcinoma *in situ* or from healthy individuals. Samples had been collected previously and stored frozen under research protocols approved by the Institutional Review Board of The University of Texas M.D. Anderson Cancer Center.

Ii-Key-linked peptides. The Ii-Key-HER-2 fusion peptides were designed by Dr Robert E. Humphreys and synthesized by Antigen Express, Inc. (Worcester, MA). The components and the coding used to describe those peptides are listed in Table I

for ease of comparison. 'Protected' peptides were created by adding an acetyl group to the N-terminus and an α -amide group to the C-terminus. Peptides were dissolved in PBS and stored frozen at -20°C until use.

CD8-activating peptides. As positive controls for E75 [HER-2 (369-377)] (KIFGSLAFL), we used HLA-A2⁺-binding peptides known to be immunogenic for human CD8⁺ cells: MART-1(27-36) (AAGIGILTV); and influenza matrix M58(58-66) (GILGFVFTL) (14-16). These peptides were synthesized as free 'unprotected' peptides by the Peptide Synthesis Core Facility of M.D. Anderson Cancer Center and purified by HPLC. Interleukin (IL)-2, IL-12, IL-4, GM-CSF, cell separation kits, were obtained from BD Biosciences Pharmingen (San Diego, CA). CD8⁺ cells were activated by established methods and E75-TCR⁺CD8⁺ cells were quantified as reported previously (17-20).

The binding potential of the p776, F7, G89 and LRMK-p776 peptides was determined with the TEPITOPE epitope-prediction software program developed by Dr Juergen Hammer (Hoffman-La Roche, Nutley, NJ) (21,22). The specificity and restriction of the peptides for MHC class II molecules was confirmed by adding Gly chains onto the N- or C-terminal to the peptide of interest and analyzing 30-amino-acid-long sequences. The MHC class II-bound peptide models were constructed as described elsewhere (19,20), with a previously reported model (23,24) used as a template.

Results and Discussion

Composition of Ii-Key-HER-2 fusion peptides. Review of the chemical analysis for all peptides (provided by Dr Humphreys) confirmed the amino acid composition described. The amounts of Ava were not quantified. A tandem mass spectrometric analysis indicated the presence of only one peak (corresponding to one charged ion) for all peptides. These peptides should have shown several peaks (corresponding to several ion species) because of the presence of arginine (R) in the peptides and in the LRMK extension. Whether the detection of only one peak was due to equipment sensitivity, small amounts of samples analyzed, or the protection afforded by the free amino and carboxy groups remains unclear.

Toxicity of Ii-Key-HER-2 peptides to PBMCs. Ii-Key peptides have been reported to be toxic in mice *in vivo* at 10-30 μ M (2). Our own tests of the peptides reported here revealed toxicity to human PBMCs *in vitro* at lower concentrations (not shown), with LRMK-p776 being more toxic than LRMK-Ava-F7 at 3000 nM. It is unclear if the toxicity was due to residual impurities from the synthesis, the presence of the C-terminal α -amide or the N-terminal basic amino acids, or overstimulation of T cells. In a population, a clone of defined specificity cannot be >4%. Decreases in PBMC numbers must be investigated in the future.

Binding affinity of LRMK-p776 to the HLA-DR. Peptides that bind to MHC class II proteins all contain a nine-amino-acid core that is extended with N- and C- terminal flanking residues that are outside the binding pocket (25). In this study, the core epitope in every HER-2 peptide studied was HER-2(780-788),

Peptide positions	Sequence	Names
Wild-type HER-2		
HER-2(777-789)	SPYVSRLLGICLT	G89
HER-2(776-788)	G-SPYVSRLLGICL	F7
HER-2(774-788)	GVG-SPYVSRLLGICL	p776
Peptides prepared by Dr Humphreys		
HER-2(774-788)	Ac- GVG-SPYVSRLLGICL-NH ₂	AE36, Ac-p776
Ii-Key+p776	Ac-LRMK- GVG-SPYVSRLLGICL-NH ₂	AE37, LRMK-p776
Ii-Key+Ava+HER-2(777-788)	Ac-LRMK-Ava- SPYVSRLLGICL-NH ₂	AE38
Ii-Key+Ava+F7	Ac-LRMK-Ava- G-SPYVSRLLGICL-NH ₂	AE47, LRMK-Ava-F7
Ii-Key+Ava+HER-2(775-788)	Ac-LRMK-Ava- VG-SPYVSRLLGICL-NH ₂	AE48
Ii-Key+Ava+p776	Ac-LRMK-Ava-GVG-SPYVSRLLGICL-NH ₂	AE39, LRMK-Ava-p776

Ac, acetyl; Ava, ε-aminovaleric acid and NH₂, α-amide.

	A.	B.
	P776: 1% threshold AYVMA-GV-GSPYVSRLLGICL	AE37: 1% threshold A-LRMK-GVGSPYVSRLLGICL
DRB	1-----10-----20	1-----10-----20
1*0101	AYVMAGVGS PYVSRLLGIC L	ALRMKGVGS PYVSRLLGIC L
1*0102	AYVMAGVGS PYVSRLLGIC L	ALRMKGVGS PYVSRLLGIC L
1*0801	AYVMAGVGS PYVSRLLGIC L	<u>ALRMKGVGS PYVSRLLGIC L</u>
1*0802	<u>AYVMAGVGS PYVSRLLGIC L</u>	<u>ALRMKGVGS PYVSRLLGIC L</u>
1*0804	AYVMAGVGS PYVSRLLGIC L	<u>ALRMKGVGS PYVSRLLGIC L</u>
*1*0806	AYVMAGVGS PYVSRLLGIC L	<u>ALRMKGVGS PYVSRLLGIC L</u>
1*1101	<u>AYVMAGVGS PYVSRLLGIC L</u>	ALRMKGVGS PYVSRLLGIC L
1*1305	AYVMAGVGS PYVSRLLGIC L	ALRMKGVGS PYVSRLLGIC L
1*1307	<u>AYVMAGVGS PYVSRLLGIC L</u>	ALRMKGVGS PYVSRLLGIC L
1*1321	<u>AYVMAGVGS PYVSRLLGIC L</u>	ALRMKGVGS PYVSRLLGIC L
	C.	
DRB	1-----10-----20-----30	
1*0101	AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L	
1*0102	AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L	
1*0404	AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L	
1*0410	AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L	
1*0701	AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L	
1*0801	<u>AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L</u>	
1*0802	<u>AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L</u>	
1*0804	AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L	
1*1305	<u>AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L</u>	
1*1321	<u>AYVMAGVGS PYVSRLLGIC LTSTVQLVTQ L</u>	

Figure 1. Potential binding affinity of HER-2 peptides to HLA-DR molecules, as determined with the TEPITOPE program. (A) p776 [HER-2(774-788); GVG-SPYVSRLLGICL] and F7 [HER-2(776-778); G-SPYVSRLLGICL]. (B) LRMK-p776 (AE37). (C) G89 [HER-2(777-789); SPYVSRLLGICLT]. Only the DR-α chain molecules that bind the HER-2 peptides with the highest affinity are shown. Italic and underlined font (L) indicates the main (P1) anchor; bolded font (Y) indicates the sequence bound; while strikethrough (X) indicates unfavorable binding to the MHC class II binding pocket.

i.e., VSRLLGICL (Fig. 1). We analyzed the potential binding affinity of the test peptides for the MHC class II HLA-DR protein by using the TEPITOPE algorithm. The molecules 'preferred' by p776, F7 and LRMK-p776 were DRB1*0102, *0801 and *0804. Since the core peptide VSRLLGICL is present in LRMK-p776, we can conclude that the binding preferences of LRMK-p776 are similar to those of p776 and F7.

Extending the p776 peptide [HER-2(774-788)] by adding LRMK did not increase the binding affinity of the hybrid peptide for any MHC class II molecule. The addition of LRMK creates an additional minimum HLA-DR-binding peptide, LRMKGVGS. This sequence is present in p776 though is absent in F7. The peptide LRMKGVGS, binds preferentially to DRB1*0806 if LRMK is proteolyzed.

The G89 peptide preferentially bound to DRB1*0404 and 0410, perhaps because the last six amino acids of G89 are part of the peptide LGICLTVQL, a nine-mer that preferentially binds DRB1*0404 and 0410. Given the predicted binding affinity of LRMK-p776 for DR1 identifying the type and amounts of cytokines produced by T cells that are activated by LRMK-p776 will be of interest. The binding affinities are neither absolute nor exclusive. Recent experimental studies have shown that p776 and LRMK-p776 bind DR4 (26,27). The important question is whether the weak and the strong binding result in distinct Th1 and Th2 cytokines or enhance the already programmed cytokines of the antigenic peptide as reported by Kallinteris *et al* (5).

Activating effects of LRMK-p776 and LRMK-Ava-F7. At 600 nM, the p776 [HER-2(774-788)] and LRMK-p776 peptides activated the proliferation and survival of PBMCs similar to that induced by F7 [HER-2(776-778)]; at 3,000 nM, proliferation and survival of the PBMCs was similar to that induced by G89 [HER-2(777-789)] (data not shown).

At 7,000 nM, the LRMK-p776 and LRMK-Ava-p776 peptides killed ~40% of the PBMCs, whereas F7 and G89 at the same concentration expanded PBMCs from the same donor by 30-40% compared with the control unstimulated PBMCs (data not shown). This finding suggests that LRMK-Ava-p776 had a higher functional avidity for TCR than F7 did, that the preparation was contaminated by some kind of toxic material, or that LRMK-p776 or LRMK-Ava-p776, at 7,000 nM, engaged many TCRs of many specificities. This last possibility would resemble the response of T cells to bacterial super-antigens such as endotoxins (28-30) in that endotoxins rapidly activate cytokine production by T cells and kill those cells shortly thereafter.

Table II. Expansion of E75-TCR⁺CD8⁺ cells from a HER2⁺ HLA-A2⁺ patient with DCIS by HER-2 peptides.

Stimulator	Cells (total)	TCR ^{Low} (% of total cells)	TCR ^{Med} (% of total cells)	TCR ^{High} (% of total cells)
None	832	46.7 (5.6)	4.7 (0.5)	1.1 (0.1)
E75 [^]	572	36.8 (6.4)	2.1 (0.3)	0.6 (0.1)
AE37 [#]	728	43.1 (6.0)	2.8 (0.4)	0.8 (0.1)
AE-47 [#]	729	45.6 (6.3)	5.3 (0.7)	1.1 (0.2)
E75 [^] + AE-37 [#]	588	13.7 (2.3)	9.2 (1.6)	2.4 (0.4)
E75 [^] + AE-47 [#]	644	25.9 (4.0)	6.9 (1.1)	1.6 (0.2)
Flu-Matrix ^{&}	812	40.2 (5.0)	1.1 (1.2)	0.5 (0.1)
MART-1 ^{&}	406	40.6 (10.0)	2.6 (0.6)	0.5 (0.1)
E75 ^{^^}	960	63.9 (6.7)	3.9 (0.4)	1.1 (0.1)
AE-37 [@]	224	7.8 (3.5)	1.2 (0.5)	0.3 (0.1)
AE-47 [@]	594	19.1 (3.2)	2.8 (0.5)	0.7 (0.1)
E75 ^{^^} + AE-37 [@]	448	29.1 (6.5)	5.4 (1.3)	1.3 (0.3)
E75 ^{^^} + AE-47 [@]	729	36.9 (5.1)	7.7 (1.1)	2.2 (0.3)
Flu-Matrix ^{**}	1,092	48.1 (4.4)	3.2 (0.3)	1.2 (0.1)
MART-1 ^{**}	945	59.9 (6.3)	6.9 (0.74)	1.8 (0.19)

Numbers represent the cell number divided by 1,000. E75 was used at 1,000 ([^]) and 5,000 nM (^{^^}). AE-37 and AE47 were used at 600 ([#]) and 3,000 nM ([@]). Flu-matrix and Mart-1 were used at [&] 1,000 and ^{**} 5,000 nM.

Table III. Free energies of the DR- α chain indicate destabilization after HER-2 peptide binding.

Position	Peptides					Changes in DR stability
	Core	F7	p776	AE-37	AE-47	
42V	-39.1	-39.5	-37.5	-38.1	-38.5	
43W ^a	-72.9	547.5	578.1	1022.7	1013.0	↓ F7/p776 vs core ↓ LMRK vs core
44R	-300.2	-294	-294	-296	-295	
45L	-41.8	-41.3	-41.6	-42.5	-42.5	
46D	-12.3	-12.3	-13.9	-15.7	-15.8	
47D	-25.2	-24.2	-25.1	-24.9	-24.5	
48F ^b	-29.9	-29.1	-29.9	81.0	84.2	Small ↓ LRMK
49G ^c	23.7	18.9	395	7918.0	7874.0	↓ ↓ ↓ with LRMK
50R ^d	-267	-264	-261	3134	3098	↓ ↓ with LRMK
51F ^e	-24	1.0	1.2	173	170	Small ↓ with LRMK
52A ^f	-17	1811	1946	3215	3182	↓ with LRMK ↓ F7/p776, LRMK vs core
53S	-30	13224	12234	13669	13914	↓ F7/p776,
54F	-56	1662	3555	3103	3095	↓ F7/p776, LRMK
55D	-18	-20	-22	-23.0	-23.8	

Core, VSRLLGICL; F7, G-SPY-core; p776, GVG-SPY-core; AE-37, LRMK-p776 and AE-47, LRMK-Ava-F7.

Induction of IFN- γ by LRMK-extended HER-2 peptides. The ability of LRMK-p776 and LRMK-Ava-F7 to induce cytokine production has been reported elsewhere (Murray *et al* unpublished data). Briefly, LRMK-p776 induced more IFN- γ in samples from more patients and more healthy donors than did LRMK-Ava-F7. Each IFN- γ induction study included IL-12 as a co-factor. In the absence of IL-12, only LRMK-p776 and LRMK-Ava-F7, among 7 peptides at very high

concentrations (e.g. 25 μ g/ml), stimulated small amounts of IFN- γ . The ability of the unprotected peptides p776, F7 and G89 to induce IFN- γ has not been tested in the same experiment.

Proliferation of E75-TCR⁺CD8⁺ cells in response to LRMK-p776 and LRMK-Ava-F7. We then sought to determine which peptide, LRMK-p776 or LRMK-Ava-F7, was more effective



SPANDIDOS PUBLICATIONS Positive free energies indicate destabilization of Met bound in LRMK and of Ser-Pro in bound HER-2 peptides.

Position	Peptides					Stability
	Core	F7	p776	AE-37	AE-47	
1L ^a	-	-	-	0.4	0.4	↓ LRMK, Ava equal
2R ^a	-	-	-	-0.2	-0.2	More stable
3M ^a	-	-	-	1.9	1.9	↓ LRMK, Ava equal
4K ^a	-	-	-	16.5	16.6	↓ LRMK, Ava equal
5G ^b	-	-	0.67	1.0	1.0	↓ Terminal GV ↓ LRMK
6G ^c	-	-	-	-	0.7	>Stable than LRMK-p776
7G ^d	-	-	-	-	1.4	Equal to AE-37
6V ^d	-	-	1.10	1.3	-	Equal to AE-47
7G ^e	-	2.2	2.20	2.5	2.5	↓ N-term peptide
8S ^e	-	12.6	10.20	12.4	12.2	↓ N-term peptide
9P ^f	-	2.2	4.80	3.9	4.0	↓ ↓ unstable proline
10Y ^g	-	2.5	0.47	0.58	0.4	Nearly equal
11V ^h	0.13	0.2	0.2	0.3	0.3	Nearly equal

Core, VSRLLGICL; F7, G-SPY-core; p776, GVG-SPY-core; AE-37, LRMK-p776 and AE-47, LRMK-Ava-F7. The free energy and stability of LRMK-p776 bound in HLA-DR and those of LRMK-Ava-F7 bound in HLA-DR are similar. The free energy is presented in Kcal/Mol.

in helping the expansion of E75-specific CD8⁺ cells in the presence of the immunogenic peptide E75 [HER-2(369-377)] (Table II). For these experiments, PBMCs from a HER-2⁺ HLA-A2⁺ patient with DCIS were activated with LRMK-p776 or LRMK-Ava-F7, in the absence or presence of E75. Controls were MART-1 and Flu-Matrix, two peptides with known immunogenicity for CD8⁺ cells. The activation doses tested were 1 μg/ml (600 nM) or 5 μg/ml (3,000 nM) for LRMK-p776 or LRMK-Ava-F7, with 1,000 or 5,000 nM, respectively, of E75.

As expected, the native E75 peptide alone activated E75-TCR⁺ cells only weakly at either concentration (1,000 and 5,000 nM) and in fact led to the decay of a substantial proportion of the E75-TCR⁺ cells. By comparison, at 1,000 nM, the control peptides Flu-Matrix and MART-1 had a moderately suppressive effect on the numbers of E75-TCR^{High} and E75-TCR^{Med} cells, though did not affect the number of E75-TCR^{Low} cells; at 5,000 nM, MART-1 led to the increase in the number of E75-TCR^{Hi} and E75-TCR^{Med} cells. The reason for these effects is unknown. Several previous studies have reported 'helper' effects by CD8⁺ cells (31). LRMK-p776 alone did not increase the number of E75-TCR⁺ cells at 600 nM and led to substantial decreases in E75-TCR⁺ cells at 3,000 nM. The LRMK-Ava-F7 peptide had similar effects. Thus it is unlikely that these two peptides would have any helper effects *in vivo* if administered alone.

Used together, however, E75 and the putative helper peptides LRMK-p776 and LRMK-Ava-F7 produced decidedly different results. At the lower doses, E75 (1,000 nM) plus LRMK-p776 (at 600 nM) expanded 4.5 times more E75-TCR^{High} cells and E75-TCR^{Med} CD8⁺ cells than did E75 alone and roughly twice as many TCR^{High} and TCR^{Med} cells as in the unstimulated condition. LRMK-Ava-F7 had similar though less potent effects compared with LRMK-p776. However, for LRMK-p776 and LRMK-Ava-F7, any increases in E75-specific cells were paralleled by decreases in overall cell

numbers: the number of E75-activated cells was 69% of the number of unstimulated cells; for E75+LRMK-p776-activated cells, 71%; and for E75+LRMK-Ava-F7-activated cells, 78%. Still more specifically, increases in E75-TCR^{Med} and E75-TCR^{High} cells were paralleled by decreases in E75-TCR^{Low} cells by 70% (for E75+LRMK-p776) and 45% (for E75+LRMK-Ava-F7).

At the higher doses, LRMK-Ava-F7 (at 3,000 nM, administered alone) was twice as strong an activator of E75-TCR^{Med} and E75-TCR^{High} cell expansion as was LRMK-p776 (also at 3,000 nM, administered alone). In terms of total cell numbers, 5,000 nM E75 (administered alone) increased the total number of cells by 15%; 3,000 nM LRMK-p776 (administered alone) decreased the total cells by 73%; and 3,000 nM LRMK-Ava-F7 (administered alone) decreased the total cells by 29% (relative to the unstimulated condition). Giving either peptide (at 3,000 nM) in combination with 5,000 nM E75 led to similar decreases in total numbers of cells, though giving LRMK-Ava-p776 (3,000 nM) plus E75 (5,000 nM) led to modest increases in the numbers of E75-TCR^{High} and E75-TCR^{Med} cells. In summary, 600 nM LRMK-p776 (administered with 1,000 nM E75) and 3,000 nM LRMK-Ava-F7 (administered with 5,000 nM E75) significantly enhanced the proliferation of E75-TCR^{Hi+Med} CD8⁺ cells.

These findings, that LRMK-p776 had a more potent 'helper' capability than did LRMK-Ava-F7, were also observed in two of the three samples tested (Murray *et al* unpublished data). In the third sample, LRMK-Ava-F7 was more potent than LRMK-p776. A similar effect has been reported independently (32). We have no explanation for these differences in potency among individuals, although it is possible that the 'helper' effects are mediated through the elimination of E75-TCR^{Low} cells and cells of unrelated specificities. Such reductions in the overall numbers of cytokine-consuming cells could make more cytokines

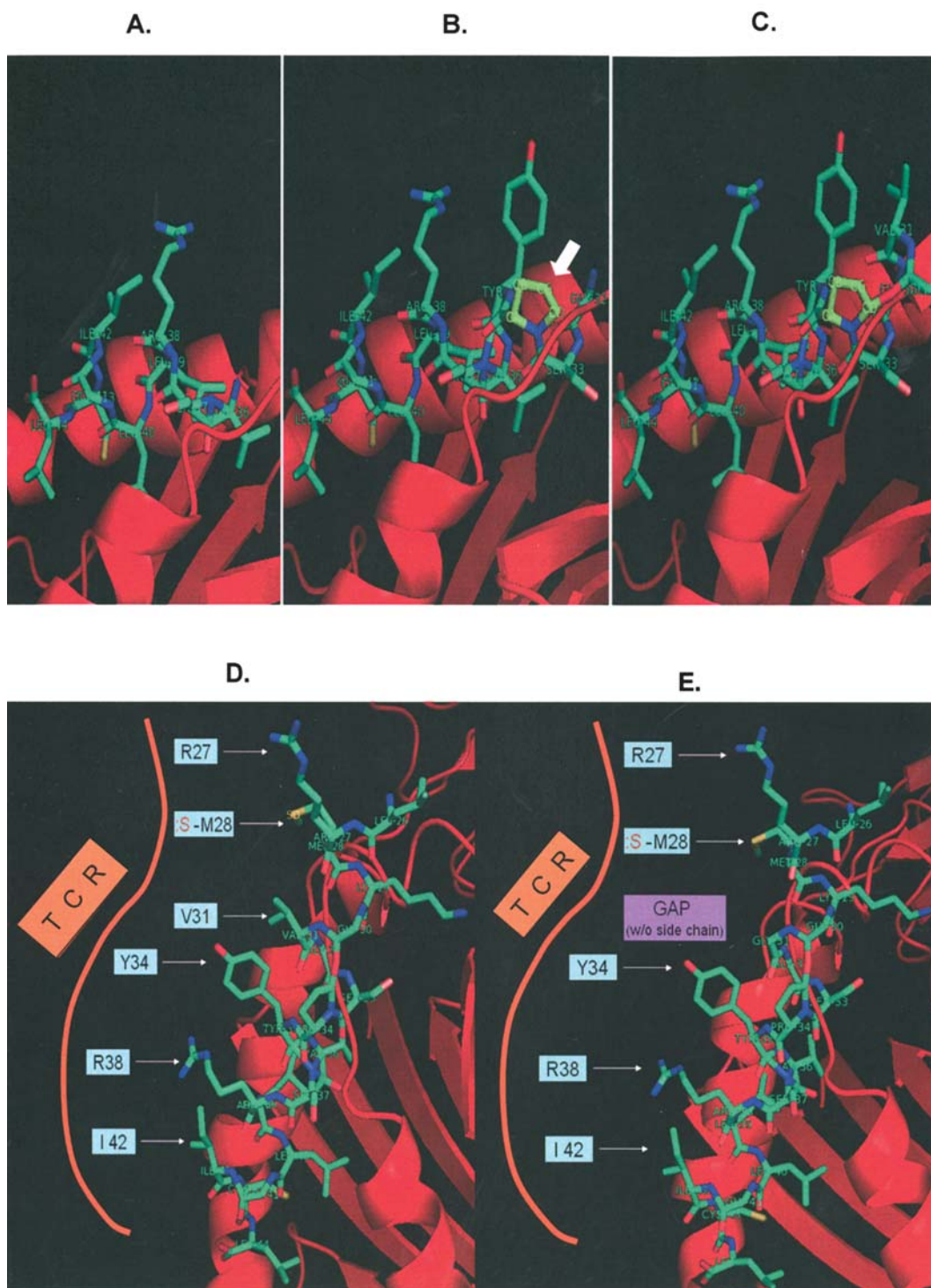



Figure 2. Molecular models of HER-2 peptides bound to HLA-DR. (A) The core epitope (VSRLLGICL). (B) F7 [HER-2(776-778); G-SPY-VSRLLGICL]. (C) p776 [HER-2(774-788); GVG-SPY-VSRLGICL]. (D) LRMK-p776 (LRMK-GVG-SPYVSRLGICL). (E) LRMK-Ava-F7 (LRMK-Ava-G-SPYVSRLGICL). HLA-DR chains are shown in red. Panels D and E are shown in side view, with the complexes rotated 90° to the left.

available to the surviving populations. Indeed, the major difference among the various treatment-condition populations may have been in the numbers of E75-TCR⁺ cells, specifically the numbers of E75-TCR^{Low} cells. In terms of the number of E75-TCR^{Low} cells, after 1 week of culture with IL-2, the unstimulated culture had 46,758 E75-TCR^{Low} cells, the culture stimulated with 1,000 nM E75 had 36,779 E75-TCR^{Low} cells, the culture stimulated with 1,000 nM E75+600 nM LRMK-p776 had 13,700 cells and the culture stimulated with 1,000 nM

E75+600 nM LRMK-Ava-F7 had 25,882 cells. E75+LRMK-bound peptide, however, did expand the number of E75-TCR^{High+Med} cells: 5,740 in unstimulated, 2,731 in E75-stimulated, 11,582 in E75+LRMK-p776-stimulated cells, 8,700 in E75+LRMK-Ava-F7 stimulated cells (Table II). When the concentrations of peptides increased, E75+LRMK-p776 expanded 6,700 E75-TCR^{High+med} cells while E75+LRMK-Ava-F7 expanded 9,900 E75-TCR^{High+med} cells. It has been reported that CD4⁺ cells are needed for a secondary

 SPANDIDOS PUBLICATIONS of CD8⁺ cells. In humans, there are always tumor specific cells (33). Additional studies are needed to clarify whether LRMK-bound HER-2 peptides help the priming of naïve CD8⁺ T cells.

Molecular models of HER-2 peptide HLA-DR. Our results indicated that at 3,000 nM, LRMK-p776 eliminated many PBMCs and that LRMK-Ava-F7 was a weaker activator than LRMK-F7. To identify the orientation of the side chains of these extended peptides when they are bound to MHC class II proteins, we built molecular models. The first model included the minimum core peptide VSRLGICL (Fig. 2A). After energy minimization, it became evident that Arg (R) and Ile (I) point upwards and thus form the potential contacts with TCR. Arg has the strongest energy of the charged N groups, though Ile can only mediate van der Waals forces. The second model, that of F7 (GSPY-VSRLGICL; Fig. 2B), shows that the tetra-peptide GSPY is positioned outside the binding pocket. The tyrosine (Y) points upwards and forms an additional potential point of contact with TCR through its H bonds and benzene ring. The third model, that of p776 (GVGSPY-VSRLGICL; Fig. 2C), is similar to that of F7 in that the hexapeptide GVGSPY is positioned outside the binding pocket. The valine (V) is oriented upward and can form van der Waals forces. However, in F7 the proline pentagonal ring (see white arrow, Fig. 2B) is exposed, although in p776 that ring is masked by valine and the free CH3/CH2 side chains point upwards.

The addition of LRMK to p776 added side chains as follows. The leucine (L) points downwards, toward the α -chain of MHC class II and could increase the stability of binding; the arginine (R) points upwards; the methionine (M) associates with the surface of the DR- α chain, exposing the nucleophilic sulfur atom; and the lysine (K) points downwards and could bind to the internal surface of the α -chain. In the LRMK-p776 peptide, the L and the R in the LRMK could collectively enhance the binding of LRMK-p776 in the inside part of the α -chain and the R and the M could enhance contact with the TCR. Hence, the side chains of LRMK form a four-fingered fork with alternating fingers Down-Up-Up-Down or MHC-TCR-TCR-MHC (Fig. 2D).

The addition of LRMK-Ava to the F7 peptide showed that the absence of the GV group removed the potential contact between valine and the TCR. In another model in which two glycines were used to model Ava, the binding structure MHC-TCR-TCR-MHC was unchanged. However, the peptide bonds C- δ \rightarrow C- α will probably affect the binding of LRMK-linked peptides to MHC class II molecules, although the nature of this change in binding remains to be determined. It is tempting to speculate that the C- δ \rightarrow C- α bonds would introduce torsion that would decrease the stability of the LRMK-MHC-II complex. This possibility might also explain the weaker stimulating ability of LRMK-Ava-F7 relative to that of LRMK-p776 which lacks Ava.

The exact role of Ava could not be determined. LRMK-Ava-HER-2(775-788) (LRMK-Ava-VG-SPYVSRLGICL) and LRMK-Ava-HER-2(774-788) (LRMK-Ava-GVG-SPYVSRLGICL, i.e., LRMK-Ava-p776) did not induce IFN- γ production, although LRMK-p776 and LRMK-Ava-F7 did. Ava did not affect the stability of peptide MHC-II

complex when modeled as glycine. Additional studies are needed in order to define the role of Ava in LRMK activity.

An extension of the core peptide successively with four, six, nine, or ten amino acids increased the positive free energy of the DR- α chain in region 43-54 and of the peptide in the sequence GSP (Tables III and IV), thereby decreasing its stability. Since the TCR contacts the peptide and the MHC class II chains, the 'opening' of the DR- α chain could affect the contact of the peptide with the TCR in ways yet to be determined.

Lys in LRMK significantly decreased the stability of the bound peptide and was followed by Met. In contrast, Leu had a weak destabilizing effect, while Arg increased the peptide stability of binding. It is then Arg, which our model finds to face TCR also a contact for HLA-DR as suggested (13).

Our findings of the presence of two minimal HLA-DR binding peptides in LRMK-p776, of complementary function by LRMK-p776 and LRMK-Ava-F7 and of variation in the conformation of the DR- α chain and peptide and orientation of side chains may be useful for enhancing the help and local inflammation during metastasis-protective cancer vaccination. If the LRMK-extended p776 is shown to function similarly with superantigens, it is then LRMK-Ava-F7, LRMK-F7, or F7 the recall 'helper' Ag which can avoid protracted suppression and tumor growth (29). Extensive studies are needed to address these questions.

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References

- Adams S and Humphreys RE: Invariant chain peptides enhancing or inhibiting the presentation of antigenic peptides by major histocompatibility complex class II molecules. *Eur J Immunol* 25: 1693-1702, 1995.
- Adams S, Albericio F, Alsina J, Smith ER and Humphreys RE: Biological activity and therapeutic potential of homologs of an Ii peptide which regulates antigenic peptide binding to cell surface MHC class II molecules. *Arzneimittelforschung* 47: 1069-1077, 1997.
- Xu M, Jackson R, Adams S and Humphreys RE: Studies on activities of invariant chain peptides on releasing or exchanging of antigenic peptides at human leukocyte antigen-DR1. *Arzneimittelforschung* 49: 791-799, 1999.
- Humphreys RE, Adams S, Koldzic G, Nedelescu B, von Hofe E and Xu M: Increasing the potency of MHC class II-presented epitopes by linkage to Ii-Key peptide. *Vaccine* 18: 2693-2697, 2000.
- Kallinteris NL, Lu X, Wu S, Hu H, Li Y, Gulfo JV, Humphreys RE and Xu M: Ii-Key/MHC class II epitope hybrid peptide vaccines for HIV. *Vaccine* 21: 4128-4132, 2003.
- Tuttle TM, Anderson BW, Thompson WE, *et al*: Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. *Clin Cancer Res* 4: 2015-2024, 1998.

7. Disis ML, Bernhard H, Shiota FM, *et al*: Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood* 88: 202-210, 1996.
8. Fisk B, Hudson JM, Kavanagh J, Wharton JT, Murray JL, Ioannides CG and Kudelka AP: Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. *Anticancer Res* 17: 45-53, 1997.
9. Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD and Cheever MA: Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. *J Immunol* 156: 3151-3158, 1996.
10. Disis ML, Grabstein KH, Sleath PR, *et al*: Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin Cancer Res* 5: 1289-1297, 1999.
11. Kuerer HM, Peoples GE, Sahin AA, *et al*: Axillary lymph node cellular immune response to HER-2/neu peptides in patients with carcinoma of the breast. *J Interferon Cytokine Res* 22: 583-592, 2002.
12. Knutson KL and Disis ML: IL-12 enhances the generation of tumour antigen-specific Th1 CD4 T cells during *ex vivo* expansion. *Clin Exp Immunol* 135: 322-329, 2004.
13. Gillogly ME, Kallinteris NL, Xu M, Gulfo JV, Humphreys RE and Murray JL: Ii-Key/HER-2/neu MHC class-II antigenic epitope vaccine peptide for breast cancer. *Cancer Immunol Immunother* 53: 490-496, 2004.
14. Coulie PG, Brichard V, Van Pel A, *et al*: A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* 180: 35-42, 1994.
15. Fisk B, Blevins TL, Wharton JT, *et al*: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 181: 2109-2117, 1995.
16. Peoples GE, Gurney JM, Hueman MT, *et al*: Clinical trials results of a Her-2/neu (E75) vaccine to prevent recurrence in high-risk breast cancer patients. *J Clin Oncol* 23: 7536-7545, 2005.
17. Lee TV, Johnston DA, Thomakos N, *et al*: Helper peptide G89 (HER-2:777-789) and G89-activated cells regulate the survival of effectors induced by the CTL epitope E75 (HER-2, 369-377). Correlation with the IFN- γ :IL-10 balance. *Anticancer Res* 22: 1481-1490, 2002.
18. Ko BK, Efferson CL, Kawano K, Kuerer HM, Sahin A, Murray JL and Ioannides CG: Stimulation of cells from a non-invaded and an invaded lymph node with a HER-2⁺ tumor with peptides corresponding to T-cell epitopes E75 and G89 induced expansion of central memory cells (TCM) from the metastasis-negative lymph nodes. *Int J Oncol* 24: 1413-1418, 2004.
19. Castilleja A, Carter D, Efferson CL, *et al*: Induction of tumor-reactive CTL by C-side chain variants of CTL epitope HER2/neu proto-oncogene (369-377) selected by molecular modeling of the peptide: HLA-A2 complex. *J Immunol* 169: 3545-3554, 2002.
20. Kawano K, Efferson CL, Peoples GE, Carter D, Tsuda N, Murray JL and Ioannides CG: Sensitivity of undifferentiated, high-TCR-density CD8⁺ cells to methylene groups appended to tumor antigen determines their differentiation and death. *Cancer Res* 65: 2930-2937, 2005.
21. Bian H and Hammer J: Discovery of promiscuous HLA-II-restricted T cell epitopes with TEPITOPE. *Methods* 34: 468-475, 2004.
22. Bian H, Reidhaar-Olson JF and Hammer J: The use of bioinformatics for identifying class II-restricted T-cell epitopes. *Methods* 29: 299-309, 2003.
23. Zavala-Ruiz Z, Sundberg EJ, Stone JD, *et al*: Exploration of the P6/P7 region of the peptide-binding site of the human class II major histocompatibility complex protein HLA-DR1. *J Biol Chem* 278: 44904-44912, 2003.
24. Zavala-Ruiz Z, Strug I, Walker BD, Norris PJ and Stern LJ: A hairpin turn in a class II MHC-bound peptide orients residues outside the binding groove for T cell recognition. *Proc Natl Acad Sci USA* 101: 13279-13284, 2004.
25. Hennecke J and Wiley DC: Structure of a complex of the human T cell receptor (TCR) HA1.7, influenza hemagglutinin peptide, and major histocompatibility complex class II molecule, HLA-DR4 (DRA*0101 and DRB1*0401): insight into TCR cross-restriction and alloreactivity. *J Exp Med* 195: 571-581, 2002.
26. Salazar LG, Fikes J, Southwood S, *et al*: Immunization of cancer patients with HER-2/neu-derived peptides demonstrating high-affinity binding to multiple class II alleles. *Clin Cancer Res* 9: 5559-5565, 2003.
27. Voutsas IF, Gritzapis AD, Mahaira LG, Salagianni M, Hofe EV, Kallinteris NL and Baxeavanis CN: Induction of potent CD4⁺ T cell-mediated antitumor responses by a helper HER-2/neu peptide linked to the Ii-Key moiety of the invariant chain. *Int J Cancer* 121: 2031-2041, 2007.
28. Dellabona P, Peccoud J, Kappler J, Marrack P, Benoist C and Mathis D: Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* 62: 1115-1121, 1990.
29. Plaza R, Rodriguez-Sanchez JL and Juarez C: *Staphylococcal enterotoxin B in vivo* modulates both gamma interferon receptor expression and ligand-induced activation of signal transducer and activator of transcription 1 in T cells. *Infect Immun* 75: 306-313, 2007.
30. Wang L, Zhao Y, Li Z, *et al*: Crystal structure of a complete ternary complex of TCR, superantigen and peptide-MHC. *Nat Struct Mol Biol* 14: 169-171, 2007.
31. Mailliard RB, Egawa S, Cai Q, *et al*: Complementary dendritic cell-activating function of CD8⁺ and CD4⁺ T cells: helper role of CD8⁺ T cells in the development of T helper type 1 responses. *J Exp Med* 195: 473-483, 2002.
32. Sotiriadou NN, Kallinteris NL, Gritzapis AD, *et al*: Ii-Key/HER-2/neu(776-790) hybrid peptides induce more effective immunological responses over the native peptide in lymphocyte cultures from patients with HER-2/neu⁺ tumors. *Cancer Immunol Immunother* 56: 601-613, 2007.
33. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG and Schoenberger SP: CD4⁺ T cells are required for secondary expansion and memory in CD8⁺ T lymphocytes. *Nature* 421: 852-856, 2003.