N-Terminally LRMK-linked HER-2 peptides, AE-37 [p776(774-788)] and AE-47 [Ava-F7(776-788)], Aid Differentiation of E75-TCR+CD8+ Cells to Perforin-positive Cells

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Abstract. *The objective of this study was to discover whether the peptides LRMK and LRMK-Ava linked to the N-terminus of peptides HER-2 (774-788) and HER-2 (776-788), respectively, help differentiation of E75-TCR+CD8+ cells. Activation was quantified in terms of proliferation of E75-TCR+CD8+ cells expressing high, medium and low density amounts of the specific TCR. Differentiation to functional CD8+ cells was quantified as induction of Perforin (Perf), the lytic-enzyme which mediates the effector function of CD8+ cells, in E75-TCR+CD8+ cells. Peripheral blood mononuclear cells (PBMCs) of 3 patients activated with E75+AE-37 and E75+AE-47 more greatly increased the number of E75-TCR^{Hi} CD8+Perf+ cells than PBMCs activated by AE-47 alone or AE-47+ E75. E75 plus cytokines and cytokines alone activated more E75-TCR^{Low} cells than did AE-37 and AE-47. E75+ AE-37 and AE-37 also induced differentiation of small- and medium-size activated CD8+ cells from BRC ascites, in allogeneic activation, to Perf+ cells. Preferential differentiation of E75-TCR+CD8+Perf+ cells in distinct patients by AE-37 and AE-47 indicates that cancer vaccines will benefit from such correct individual and disease-associated help. Additional studies using the natural peptides p776 and F7 are needed to understand whether the LRMK-(Ava) tetra-, or pentamer augments or inhibits differentiation of CD8+ cells, compared with native, natural HER-2 peptides and/or protects CD8+ cells activated by E75 and by other HLA-I bound peptides from death. Our findings also develop a model for uniform quantification of differentiated CD8+ effectors.*

*Both authors contributed equally to this work.

Abbreviations: Ii, invariant chain; PBG, peptide-binding groove; Ava, ε-aminovaleric acid; BRC, breast cancer; G89, HER-2 (777-789); AE37, LRMK-HER-2 (774-788); AE47, LRMK-Ava-HER-2 (776-788); TCR, T-cell receptor; MFI, mean channel fluorescence; x2, y2: geometrical means of channel fluorescence; Perf, Perforin; LU, lytic units; li-Key: LRMK.

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We report our findings on help for differentiation of CD8+ cells from breast cancer (BRC) patients by protected-Ii-key (LRMK)-linked p776 and Ava-F7. The term protected is used by Generex (Toronto, Ontario, Canada) to indicate that peptides used in this and other studies were *N*-terminally acetylated and *C*-terminally amidated. Addition of the acetyl and amide groups was expected to protect the peptide from exopeptidases. It is unclear whether the acetyl and amide groups hydrolyze at neutral pH 7.4-7.6 and the peptide is unprotected, or they do not hydrolyze and continue to protect peptides p776 and F7. In the first scenario, the LMRK-linked HER-2 peptide is a basic peptide. In the latter scenario, the acetyl and amide groups can form novel epitopes which activate CD8+ or CD4+ cells. The two HER-2 peptides 776 and F7 have been previously reported. F7 activated *in vitro*

peripheral blood mononuclear cells (PBMCs) from ovarian and BRC patients. F7, HER-2 (776-788) was identified by Drs. Fisk and Ioannides (1, 2). p776, HER-2 (774-788, or 776-790) was identified by Dr. Disis and Cheever's laboratories (3, 4). F7 and p776 have the same *C*-terminus; F7 is shorter than p776 at the *N*-terminus by two amino acids (Gly-Val). F7 was confirmed by Drs. Papamichail and Baxevanis (5) as naturally processed and presented by several human MHC-II molecules.

The LRMK-Ava extension was identified by Dr. Ioannides in 2000-2001, as the most activating *N*-terminal fragment of the human Ii-chain from a group of 4-5 *N*-terminal Ii-chain fragments linked to G89 (6). The activating function of LRMK-Ava was demonstrated in a detection system with a novel and sensitive methodology (6). Previous studies on function of LRMK and other related compounds measured the potential co-activation of proliferation by Ii-chain fragments of a murine hybridoma expressing a T-cell receptor (TCR)-specific for the pigeon cytochrome-c. The read-out was the proliferation of IL-2 dependent T-cells over a range of dilutions. That method, which is not quantitative, found LRMK-Ava less activating than other Ii-human *N*-terminal peptides (7-9). That approach is not sensitive enough when responders are polyclonal human populations with numerous distinct specificities and distinct HLA-DR molecules. It is unclear if the Ii-chain always contains leucine or more often tyrosine.

The LRMK sequence followed by a proline-rich sequence is proteolyzed to allow binding of CLIP peptide to HLA-DR (10). Activation of T-cells by the LRMK peptide linked to HER-2 peptides has not been previously tested in humans. IFN-γ was measured in PBMCs from several healthy donors and from several patients activated each by 6 HER-2 peptides, by ELISPOT. PBMCs were stimulated 3 times in a row with the 6 peptides, and then the responders were tested for recognition of the "epitope only" peptide AE-36 by ELISPOT.

The task of finding a function for LRMK in humans presented us with extreme complexity for several reasons. (i) 5 of the peptides to be tested contained Ava, while the sixth (AE-37) did not; we were not provided control-peptides; (ii) All peptides including the AE-36, "epitope only", were listed as being *N*-acetylated and *C*-terminally amidated; (iii) Production of IFN-γ, did indicate a function, since quantification of other cytokines was not made. The balance between Th1: Th2: T_{reg} cytokines determine the type of CD4 response; (iv) Since all peptides were acetylated and amidified, we could not exclude that T-cells which recognize a novel epitope formed by LRMK or LRMK-Ava would be activated instead of the HER-2-specific cells; (v) repeated T-cell activations usually result in activation-induced cell death of high affinity clones, and survival of low affinity clones.

To quantify responses to the LRMK fragments, we used a more sensitive IFN-γ-ELISA, and modifications of an activation method which we have developed (11-14). We quantified the IFN-γ response of T-cells from 6 healthy donors and 11-13 BRC patients, at priming and re-activation using our sensitive quantitative approaches. We found that of the 7 peptides tested, AE-37 was immunodominant in that it activated higher amounts of IFN-γ than the other 5 peptides in more patients. Surprisingly, the "non-protected and non-LRMK-peptide-extended', G89, activated IFN-γ, with somewhat lower frequency than AE-37 but higher than the one anticipated for AE-39 and AE-47. Even more surprising, AE-37 and G89 were complementary activators because each activated T-cells from patients not activated by the other. G89 is shorter than p776, with 3 amino acids at its *N*-terminus (Gly-Val-Gly), and has an additional amino acid at *C*-terminus (Thr) (10).

Differentiation of cytolytic CD8+ cells in response to TCR signals is a two-step process. First, CD8+ cells activate synthesis of IFN-γ, whose secretion requires IL-12 as cofactor. Second, IFN-γ-positive cells activate synthesis of Perforin, whose secretion requires IL-2 as cofactor. Cells which produce high amounts of IFN-γ, die by apoptosis at re-activation, thus production of perforin is detected either at priming or the latest, at recall, for low-affinity CD8+ cells which do not activate IFN-γ synthesis at priming (14).

Materials and Methods

Patients. PBMCs and malignant ascites were obtained from 4 BRC patients. All patients and healthy volunteers signed informed consent to participate in the study, under research protocols approved by MDACC.

Peptides and reagents. The Ii-chain peptides have been reported elsewhere (7-9). LRMK-Ava was found to be the less toxic and more activating from a group of 4-5 LRMK-containing peptides synthesized as one peptide with G89 (6). Peptides had *N*- and *C*-termini protected. Mass spectrometry-sequencing showed one peak instead of 3-4 (10). G89 and E75 (369-377) were synthesized as free peptides and purified by the Core Facility of MDACC (11, 12, 16). All reagents were from BD PharMingen (San Diego, CA, USA).

T-cell activation. A volume of 30-40 ml (5 green-cap tubes or less) of blood was drawn from 3 patients. The blood was delivered immediately to the laboratory. PBMCs were separated, stained for HLA-A2 expression and cultured immediately to minimize losses from freezing (Murray *et al*., submitted for publication, 2008). AE-37, AE-47, E75, AE-37+E75, AE-47+ E75 were added to PBMCs and tumor associated lymphocytes (TAL) in various concentrations following procedures modified for this study (11-14, 16). Control helper peptides and negative control HLA-DR peptides were not added. Negative control cytokines alone were added in parallel in separate cultures. Quantification of E75-TCR+CD8+Perf+ cells (Figure 1) used modifications of our methods required by this study (14).

Quantification. To develop a homogenous quantification system, for all patients, which takes into account the cell number and the amount of Perforin per cell, we devised lytic units (LU). The lytic
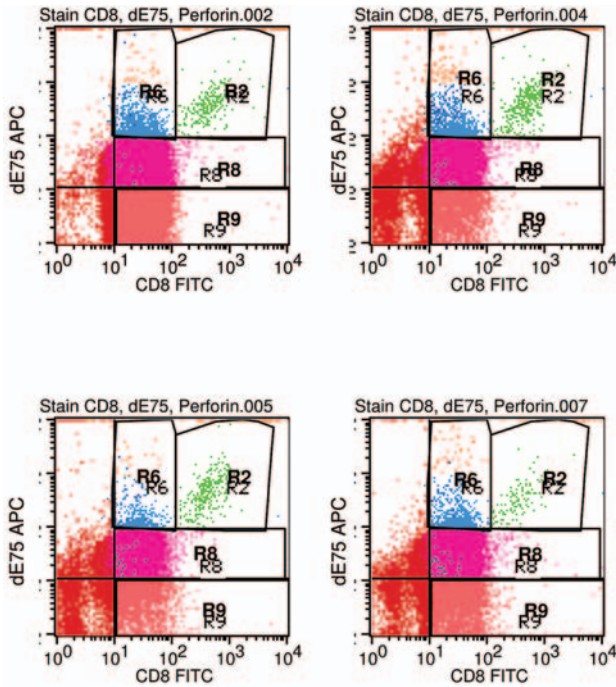
Figure 1. *E75-TCR^Med cells were present in the small and medium BRC-TAL activated by E75 and AE-37. A, BRC-TAL activated by cytokines and allo-APC, THP-1. Upper left (UL) quadrant, E75-TCR⁺ CD8⁻=11.99%; lower left (LL) quadrant E75-TCR⁻CD8⁻=4.05%; lower right (LR) quadrant E75-TCR⁻CD8⁺=14.53%; upper right (UR) quadrant E75-TCR⁺CD8⁺ quadrant contains populations R2, R6 and R8 (=69.42%) of total cells, R2=0.19%, R6=1.39%, R8=68.28%. Cells gated=115,252. B, BRC-TAL activated by cytokines, allo-APC, THP-1 pulsed with AE-37+E75. UL=18.41%; LL=4.81%; LR=6.34%; UR=70.43% of gated cells, R2=0.45%, R6=2.84%, R8=67.33%. Cells gated=81,560. C, BRC-TAL activated by cytokines, allo-APC, THP-1 pulsed with AE-37. UL=19.97%; LL=6.63%; LR=6.34%; UR=66.16% of gated cells, R2=0.55%, R6=1.86%, R8=63.95%. Cells gated=51,360. D, BRC-TAL activated by cytokines, allo-APC, THP-1 pulsed with E75. UL=15.71%; L =3.29%; LR=6.67%; UR=74.33% of gated cells, R2=0.011%, R6=2.02%, R8=72.57%. Cells gated=110,939.*
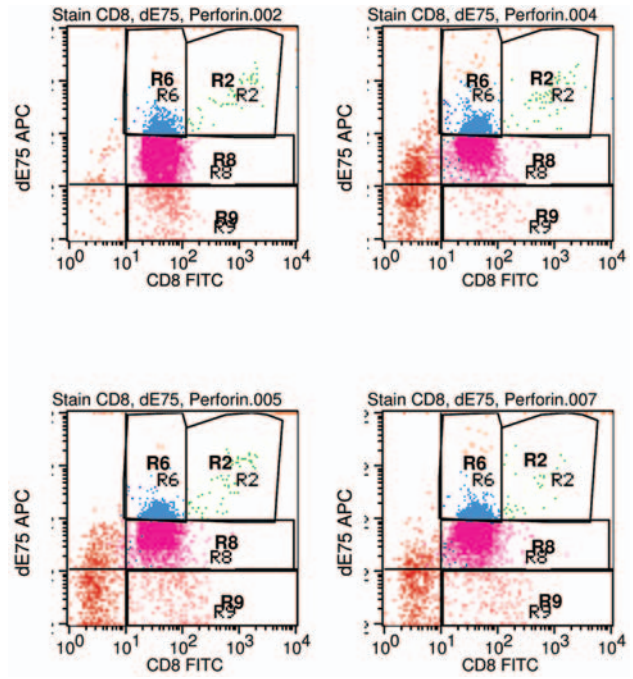
Figure 2. *E75-TCR^Med cells were present in the large BRC-TAL activated by E75 and AE-37. A. BRC-TAL activated by cytokines, allo-APC, THP-1. UL=0.39%; LL=0.20%; LR=2.21%; UR=96.49% of gated cells, R2=0.49%, R6=12.51%, R8=83.70%. Cells gated=12,325. B. BRC-TAL activated by cytokines, allo-APC, THP-1 pulsed with AE-37+E75. UL=4.72%; LL=3.01%; LR=1.10%; UR=91.17% of gated cells, R2=0.86%, R6=28.64%, R8=60.80%. Cells gated=9,660. C. BRC-TAL activated by cytokines, allo-APC, THP-1 pulsed with AE-37. UL=2.46%; LL=4.24%; LR=2.68%; UR=90.62% of gated cells, R2=0.69%, R6=20.61%, R8=68.38%. Cells gated=9,436. D. BRC-TAL activated by cytokines, allo-APC, THP-1 pulsed with E75. UL=1.55%; LL=2.50%; LR=1.51%; UR=94.44% of gated cells, R2=0.27%, R6=25.50%, R8=68.97%. Cells gated=13,463.*

units were obtained by multiplying the percentage E75-TCR⁺CD8⁺ cells (α) with the percentage (sub-numeral) of E75-TCR⁺CD8⁺Perf⁺ cells ($0.\beta$) in each population of TCR^Hi, Med and Low cells ($0.\gamma^{TCR}$) and with the geometrical channel mean fluorescence index (MFI) of Perf ($\delta^{MFI.Perf}$) in each population. *i.e.* $LU = \alpha \times 0.\beta \times 0.\gamma\ TCR \times 0.\delta\ MFI.Perf.$

The "help" by AE-37 and AE-47 was considered significant if the LU in populations activated by AE-37 and AE-47 was 2-fold or higher than the LU in populations activated by cytokines alone. Cytokines were used at the same concentration in cultures with or without peptide. Our quantification approach can be extended by including additional individual parameters of effectors such as cell size and indicator effector-function quantified from responses to a standard BRC cell line agreed by consensus.

Results

The minimum-length peptide bound in the peptide-binding groove (PBG) of HLA-DR is 9 amino acids long (17, 18). The minimum HLA-DR-bound HER-2 peptide from the area 770/772-789/791, predicted by algorithms, is 780-788: VSRLLGICL (19, 20). F7 (HER-2, 776-788), GSPYV SRLLGICL contains the flanking (outside the groove) peptide, GSPY (19). The flanking peptide of p776 is GVGSPY. When penta-peptide LRMK-Ava is added to the *N*-terminus of F7, the length of the flanking outside-chain increase to the 9 amino acids: LRMK-Ava-GSPY. When tetra-peptide LRMK is added

to p776, the length of the flanking chain increase to the 10 amino acids. LRMKGVGSPY. The distance of LRMK from the HLA-DR-PBG increases from AE-47 to AE-37 by one amino acid. We investigated whether the difference between sequences K-GVG and K-Ava-G affected the help given by AE-37 and AE-47 peptides for E75-TCR-specific CD8$^+$ cell differentiation.

Patient 1. BRC-TAL.11/2004. To identify whether AE-37 helps E75-specific CTL expansion and differentiation, activated IL-2 was used to expand TAL with HLA-A2$^+$ THP-1 cells as APC. THP-1 cells were pulsed with 25 μg of AE-37 alone or together with E75. This is an allogeneic T-cell activation system. Most T-cells are expected to recognize distinct peptides than E75. After re-activation with 5 μg of AE-37 and E75, TAL were cultured for one more week, and then analyzed for the presence of E75-TCR$^+$CD8$^+$Perforin$^+$ cells.

First analysis. More than 400,000 cells were stained and when possible more than 100,000 cells were analyzed. Only populations of T-cells activated by AE-37 and AE-37+E75 were smaller in number than the control and E75-only activated T-cell populations (Figure 1). Analysis was performed with live cells of small and medium size. Cells with forward scatter (FS) (200-450) were considered as being of small size and cells with FS (450-800) as cells of medium size. CD8$^+$ cells comprised more than 20% of total small and medium-size cells. The majority of T-cells were E75-TCR^{Low}CD8^{Low}. Peptides were immunogenic as shown by the decrease in the population of E75-TCR$^-$CD8^{Low} cells (Figure 1), and the increase in the population of E75-TCR^{Low}CD8^{Low} cells from 50.5% (NP-activated cells stained with negative control antibodies), to 68.3% (NP-activated), 67.33% (AE-37+E75), 64.0% (AE-37) and 72.6% (E75). The percentage of Perf$^+$ cells (around 90%) was similar in all populations. x^2 MFI (Perforin) ranged between 19 to 23 in E75-TCR^{Low}CD8^{Low} cells and was 27 in isotype control-only stained cells (Figure 2).

We found two small E75-TCR^{Med}CD8$^+$ populations in BRC-TAL. One, CD8^{Hi} cells was designated as R2 and the the second, CD8^{Low} cells, as R6. R6 was larger in numbers than R2. Surprisingly, cells in population R2 increased more at activation with AE-37+E75 and AE-37 alone than did R6 cells. Specifically, the percentage of E75-TCR$^+$CD8$^+$ cells increased by 2.2 and 2.8 times and by 4 and 5 times compared with cells activated by cytokines only and E75, respectively (Table I A).

R2-CD8^{Hi} cells expressed similar amounts of perforin/cell regardless of the activator. The major difference in responders was that AE-37 expanded 2-fold more E75-TCR^{Hi}CD8^{Hi}Perf^{Hi} cells than the other activators. In contrast, R6 cells expressed on average less perforin per cell than did R2 cells. The differences in numbers of E75-TCR^{Hi}CD8^{Low}Perf^{Low} cells

were not significant. Since AE-37+E75 expanded more cells than did the other peptides, the LUAE-37 were 2-times higher than the LU$_{NP}$ activated by control NP. Our study demonstrated that AE-37 helps expansion and differentiation of E75-TCR^{Hi}CD8^{Hi}Perf^{Hi} cells in the small and medium-size populations (Table I B, C).

Second analysis. Because the number of perforin-positive cells was low in the cells of small plus medium-size, responses of cells of large size (FS, 800-1000) were investigated. The large-size cells correspond to activated lymphoblasts. The large-size cells comprised 5.8% (NP sample) and 10.0% (AE-37 sample). E75-TCR$^+$ cells comprised 90-94% of the total large-size cells. The size of the R2 population was similar in small, medium and large T-cells.

Surprisingly, population R6 (E75-TCR^{Med}CD8^{Low}) was larger in large-size R6 cells. It comprised 5.5% (blank), 12.5% (NP-activated), 28.6% (AE-37+E75), 20.6% (AE-37) and 25.5% (E75). Our results show a cumulative effect of AE-37 and E75 in the increase in E75-TCR^{Med} cells. The amount of Perforin was similar and low in all samples in the R6 population. The amount of perforin in R2 populations was as follows: NP-activated (25% and 261-x^2 MFI), AE-37+E75 (20.4% and 167-x^2 MFI), AE-37 (33.9% and 158-x^2 MFI), and E75-activated (13.9% and 114-x^2 MFI). Therefore, the effector function of large E75-TCR$^+$CD8^{Low} cells will depend on higher numbers of effectors (2-3 times higher) expanded by E75 and AE37$^+$ E75 but not on their higher amount of perforin.

Patient 2. BRC-PBMCs. Re-activation by AE-37 and AE-47 reduced the number of live E75-TCR$^+$CD8$^+$ cells compared with cytokine-only cultured cells. Fewer AE-47-, and AE-37-activated E75-TCR$^+$ cells survived than E75-activated cells; more AE-47+E75-activated E75-TCR$^+$ cells survived than AE-37+E75-activated E75-TCR$^+$ cells (10) (Table II). The MFI of E75-TCR$^+$ cells activated by AE-47+E75 and AE-37+E75 were 2.3 and 4.1 times higher than the MFI of E75-TCR+ cells activated by E75 plus cytokines or cytokines only. The MFI of CD8 activated by AE-37+E75 was 2 times higher than the MFI of CD8$^+$ cells activated by E75. Therefore, AE-37 primarily, and AE-47 secondarily, alone, or together with E75, protected more cells with high-density of E75-TCR and CD8 per cell than E75 alone (Table II). Such cells can recognize smaller numbers of E75-HLA-A2 complexes on tumors. Only cells activated by AE-37+E75, AE-47 and AE-47+E75 had 2-fold more perforin per cell than E75-only activated cells. (Table III).

Patient 3. BRC-PBMC. 3-4/2006. PBMCs were activated from another patient Results of activation were measured on 4/10/2006. Cells were activated with 10 μg of AE-37, or AE-47 alone or together with 10 μg/ml E75. E75-TCR$^+$ cells were not expanded by AE-37 or AE-47. However, 2-fold

Table I. *A. E75-TCR$^+$ CD8$^+$ small- and medium-size cells after activation of PBMCs from Patient 1 in R2 and R6 populations.*

Population	NP			AE37+E75			AE-37			E75		
	(%)	CD8 (MFI)	TCR (MFI)	(%)	CD8 (MFI)	TCR (MFI)	(%)	CD8 (MFI)	TCR (MFI)	(%)	CD8 (MFI)	TCR (MFI)
R2-CD8^{Hi}	0.18	462	408	0.40	510	455	0.51	433	404	0.10	395	389
R6-CD8^{Low}	1.17	25	155	2.41	24	159	1.50	23	142	1.56	27	138
Fold-increase												
R2	1.0			2.22			2.83			0.55		
R6	1.0			2.06			1.29			1.33		

AE-37 and E75 are the activating antigens. NP indicates cells not activated with peptides. LU: lytic units. Mean MFI indicates the geometrical mean MFI of CD8 and of E75-TCR respectively. MFI of TCR and CD8 in resulting cells did not increase after peptide activation. Fold-increase is the percentage of double positive cells resulting after two consecutive activations divided by that of double positive cells in cultures not activated by peptides. (%) indicate the % CD8$^+$ cells. 100,000 to 150,000 cells were collected and counted by flow-cytometry for this analysis. Activation with AE-37 reduced the total number of cells by at least 40%, while activation with AE-37 + E75 reduced the number of cells by 30%. All MFI values are geometrical means.

B. E75-TCR$^+$Perf$^+$ small- and medium-size cells in R2-CD8^{Hi} population.

TCR level	Med		Hi	
	(%)	MFI-Perf (LU)	(%)	MFI-Perf (LU)
NP	92.6	73	7.4	116
		12.2		1.5
AE-37+ E75	90.2	75	9.8	112
		27.1		4.4
AE-37	85.1	62	14.9	99
		26.9		7.5
E75	92.5	63	7.48	130
		5.82		1.0
Fold increase		2.22		5.0

TCR^{Low} cells were not present.

C. E75-TCR$^+$ Perf$^+$ cells in R2-CD8^{Low} population.

TCR level	Low		Med		Hi	
	(%)	MFI-Perf (LU)	(%)	MFI-Perf (LU)	(%)	MFI-Perf (LU)
NP	0.38	53	93.5	32	2.68	28
		0.2		35		0.9
AE-37+ E75	0.31	66	86.9	34	4.23	24
		0.5		73		2.44
AE-37	0.52	30	90.4	25	2.6	26
		0.24		34		1.0
E75	0.64	53	94.1	30	2.49	27
		0.52		44		1.0
Fold increase	n/a		n/a		2.4	

more E75-TCR$^+$CD8$^+$ cells survived and 1.5 times more E75-TCR^{Hi}CD8$^+$Perf^{Hi} cells survived when activated by AE-37+E75 than by AE-47+E75 or E75 alone (Table IV).

Patient 4. 5/8/2006.The next activation experiment was very large, with AE-37 and AE-47 tested at 2 different concentrations and irrelevant CD8$^+$ activated peptides used for activation, for reasons unknown. Peptides activating CD4 cells or dominant HER-2 helper peptides G89 and F13 would have been reliable controls.

AE-37 and AE-47 at 1 and 5 μg helped expand similar numbers of E75-TCR$^+$CD8$^+$ cells from PBMCs from patient 4; 1 μmg/ml AE-37 + 1 μg/ml E75 was a stronger helper than 1 μg/ml AE-47 + 1 μg/ml E75 because it expanded more E75-TCR$^+$CD8$^+$ cells; 5 μg/ml AE-47 + 5 μg/ml E75 was a stronger helper than 5 μg/ml AE-37 + 5 μg/ml E75 because it also expanded more E75-TCR$^+$CD8$^+$ cells.

Table II. *E75-TCR$^+$CD8$^+$ cells in Patient 2. Re-activation.*

	E75-TCR$^+$CD8$^+$ (%)	MFI	MFI (E75-TCR)
NP	1.12	145	135
AE-37	1.33	177	264
AE-37 + E75	1.40	334	475
AE-47	1.62	178	228
AE-47+ E75	1.05	213	348
E75	0.78	159	105

(%) E75-TCR$^+$CD8$^+$ in gated cells, MFI=geometrical MFI CD8$^+$ cells.

Although the percentage of E75-TCR$^+$CD8$^+$ cells was similar in both cultures the total number of live cells was 2-fold higher in cultures activated by AE-47 (F7) than in those activated by AE-37 (p776) (Table V). Comparative analysis

Table III. *LU in Patient 2 after re-activation.*

E75-TCR	AE-37 (%)	AE-37 Perf LU	AE37+E75	AE37+E75 Perf LU	AE-47 (%)	AE-47 Perf LU	AE47+E75	AE47+E75 Perf LU	E75 (%)	E75 Perf LU
Low	21.1	30	10.9	26	22.4	55	13.3	24	44.2	40
		6.3		2.8		12.3		3.2		17.8
Med	62.2	57	60.7	144	61.8	96	62.7	96	53.6	78
		35.4		87.4*		59.3		60.2		41.8
Hi	16.4	114	26.3	222	12.5	166	18.7	200	0.7	126
		18.7		58.4**		33.6		37.4		0.9
Fold increase	3.34		5.38		2.55		3.81		0.64	

NP, AE-37, A-37+E75, AE-47, AE-47+E75=% E75-TCR$^+$ cells. % Perf = Geometrical MFI–perforin. (*, **) highest significant increase. After activation with NP the % of E-75-TCR^{Low} cells and MFI^{Perf} was 38.9 and 30, the % of E-75-TCR^{Med} cells and MFI^{Perf} was 56.7 and 56, and the % of E-75-TCR^{Med} cells and MFI^{Perf} was 4.9 and 76. The corresponding LU were 11.7, 31.7 and 3.6 respectively.

Table IV. *LU in Patient 3 activation.*

Antigen	E75 %	TCR^{Low} %	TCR^{Low} Perf LU	TCR^{Med} %	TCR^{Med} Perf LU	TCR^{Hi} %	TCR^{Hi} Perf LU
NP	1.22	25.4	32	42.3	44	28.2	118
			9.9		22.7		40.6
E75	1.50	22.5	28	32.7	48	40.9	181
			9.5		23.5		11.1
AE-47	1.47	26.3	25	34.9	36	37.7	102
			12.2		18.5		74.1
AE-37	2.21	28.6	29	30.5	43	39.1	129
			18.3		29.0		111.5*
AE-37+E75	2.47	25.1	32	38.1	48	37.8	161
			19.8*		45.2*		150.3*
AE-47+E75	1.1	25.4	25	31.4	31	42.4	183
			7.0		10.7		85.4*
Fold increase (AE-37+E75 *vs*. NP)	2.03	2.00		2.00		2.10	

% E75=% E75-TCR$^+$CD8$^+$ cells in gated cells. % TCR^{Low}, TCR^{Med} and TCR^{Hi} is a % of E75-TCR$^+$CD8$^+$ cells. % Perf=Geometrical MFI–perforin.

Table V. *Patient 4, activation.*

Antigen	% Perf$^+$ TCR^{Low}	Perf (LU)	% Perf$^+$ TCR^{Med}	Perf LU	% Perf$^+$ TCR^{Hi}	Perf LU
NP	31.1	253	3.6	297	0.82	409
		527.2		71.7		22.5
E75-5	33.3	204	2.2	285	0.63	421
		516.0		47.7		20.2
AE-37-5	28.1	213	4.9	265	1.21	462
		257.4		55.9		24.0
AE-47-5	20.7	219	3.3	261	0.87	442
		181.3		34.5		15.4
AE37+E75-5	36.5	164	8.1	212	1.99	381
		508.8		146.0*		64.4*
AE47+E75-5	37.7	198	8.9	260	2.51	447
		500.1		155.0*		75.2*
E75-1	36.5	230	2.2	294	0.66	396
		554.1		42.7		17.2
AE-37-1	31.6	230	2.1	285	0.65	454
		443.3		36.5		18.0
AE-47-1	30.0	234	3.5	269	0.89	413
		484.4		65.0		25.4
AE37+E75-1	23.3	183	15.9	243	5.30	370
		187.6		170.0*		86.3*
AE47+E75-1	29.3	167	8.2	209	2.25	404
		254.5		89.1		47.3*

% E75=% E75-TCR$^+$CD8$^+$ cells in gated cells. % TCR^{Low}, TCR^{Med} and TCR^{Hi} are % of E75-TCR$^+$CD8$^+$ cells. % Perf=Geometrical MFI–perforin.

of immunogenicity was made using LU. Cumulative results (Table VI) show that AE-37 was immunodominant in all three patients, but CD8$^+$ cell differentiation to Perf$^+$ cells required both AE-37 and E75.

Discussion

The objective of this study was to identify whether peptides AE-37 and AE-47 help expansion and differentiation of HER-2 specific CD8$^+$ cells to effectors with lytic potential. This study was requested from us in 12/2004, by Dr.Murray, immediately after we completed (9/2004) our large study on identification of immunodominant HER-2 peptide linked with

LRMK, in induction of IFN-γ from 6 healthy donors and 11-13 BRC-patients (10). It was also requested that each experiment, to determine help, be performed by a different operator. Based on reports published in 2006, both our studies were necessary for IRB to commence a Phase I clinical trial with BRC (21, 22). We were not informed of Clinical Studies. None of us was included in any report or abstract published with our findings above. Therefore, our study was more '*blind*' than customary.

Table VI. *Immunodominant helper antigen for E75-TCR$^{Low, Med, and Hi}$ cells.*

Patient/ Ag concentration μg	TCR^{Low}		TCR^{Med}		TCR^{Hi}	
	Dominant	Sub-dominant	Dominant	Sub-dominant	Dominant	Sub-dominant
2 5	NP/E75	NP/E75	AE37+E75	AE-47	AE37+E75	AE-47+ E75& AE-47
3 10	AE-37+ E75/ AE-37	AE-47	AE37+E75	AE-37	AE-37+E75	AE-37
4 1	E75	NP	AE-37+E75	AE-47+E75	AE-37+E75	AE-47+E75
4 5	NP/E75	NP/E75	AE-47+E75	AE-37+E75	AE-47+E75	AE-37+E75
Cumulative	NP/E75 3 out of 4	NP 3 out of 4	AE37+E75 3 out of 4	Mixed	AE-37+E75 3 out of 4	Mixed

Our novel findings are: (i) AE-37 consistently helped differentiation of E75-TCR⁺CD8⁺ cells to Perf⁺ cells; (ii) The effect was AE-37 concentration-independent since it was observed in all four T-cell samples tested; (iii) The effect of AE-37 was also qualitative because E75-TCR^{Hi} and E75-TCR^{Med} cells proliferated more, while E75-TCR^{Low} cells either did not increase or died; (iv) AE-47 had a similar effect to AE-37, its helper effect was in general half of the effect of AE-37. In one patient, at higher concentration, AE-47 was qualitatively better than AE-37 because it eliminated fewer antigen-specific cells than did AE-37; (v) The CTL-activating peptide was a weak, if at all, activator of E75-TCR⁺CD8⁺ cells. Therefore the one amino acid shift and deletion from AE-47 to AE-37 modulated the help for CTL effector-function depending on patient and composition of the "*protected Ii-key-linked HER-2 peptide*".

E75 did not eliminate E75-TCR^{Low}CD8⁺ cells in T-cells from all patients tested. In contrast with AE-37+E75 and AE-47+E75, E75 alone eliminated E75-TCR^{Med+Hi}CD8⁺ cells. That each experiment was performed by a different operator indicated that our results describe an actual phenomenon and are not an experimental artifact.

What is the possible mechanism of selective help and simultaneous apoptosis by AE-37 and AE-47? The decrease in E75-TCR^{Med+Hi} cells observed after activation with cytokines and cytokines plus E75 suggests that antigen-specific cells decayed by themselves or they became over-activated and died. The higher density of receptor per cell transformed an activating-signal in a death-signal.

The decrease in E75-TCR^{Low} cells on activation with AE-37 and less by AE-47 cannot be explained. It is tempting to propose that AE-37 and (less so) AE-47 induce a pro-survival factor which protects few cells with TCR^{Hi} and some with TCR^{Med}, but does not protect cells with TCR^{Low}. An alternative hypothesis is that AE-37 and AE-47 contain another component in addition to the peptide listed. The additional component strongly but briefly activates cells with TCR^{Low} or all cells which will die, soon after. Then E75 activates *de novo* T-cells. The amount of cytokines induced by E75 would be sufficient to differentiate the 30-40% surviving cells. In the absence of control peptides and without determining the amount of cytokines and their receptor expression, as well as the contribution of other factors, it is impossible to explain our findings. It will be interesting to learn the effects of purified AE-37 on CD8⁺ cell differentiation now in phase II clinical trials in USA and Greece.

Quantification of the functional effectors. In a previous study we reported that AE-37, and to lesser extent, AE-47 deleted a large number of PBMCs at priming and re-activation. These studies confirm our previous findings. For example BRC-TAL decreased by more than 20% when activated by AE-47 and by 50% when activated by AE-37 (Figure 1).

The death-inducing effect was not observed at concentrations of 1 μg/ml, or 500 nM in Patient 4 but was evident at 2,500 nM. The effects were observed in a closed activation system *in vitro*. How representative is the closed activation *in vivo* for vaccines *in vivo*? To quantify the effects using the same rule, we took into account the number of antigen-specific CD8⁺ cells counted after priming, the fraction of these cells with high-density TCR molecules for the specific antigen, and the amount of Perforin in each population. We did not include the density of the specific TCR and the density of CD8.

Our approach to quantification may be developed in future clinical studies to estimate the real number of effectors activated at priming, *in vitro* and the clinical results on vaccination *in vivo*. Mathematical methods can develop algorithms to consider the role of independent and combined variables. It is useful to predict which cancer patients should be vaccinated and which should not. This information should be useful for cancer vaccine studies worldwide.

Note. After this study was terminated, stronger help for CTL effector-function by AE-47 (re-named peptide D, by Dr. Humphreys) than by AE-37 (re-named peptide F) and AE-39 (re-named peptide B) was reported in a different model (23). Although the peptide sequences published are identical to those of AE-37 and AE-47, we do not know if the composition of AE-37 and peptide F is identical. Only papers of Drs. Ioannides and Peoples and press-disclosures of Generex maintain the names AE-37 and AE-47. Financial interest and ownership of the tumor antigens used in this study and of the results of this research is described in detail in reference 10.

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