

# Peptide Priming of Cytolytic Activity to HER-2 Epitope 369–377 in Healthy Individuals<sup>1</sup>

Brett W. Anderson, George E. Peoples,<sup>2</sup>  
James L. Murray, Michael A. Gillogly,  
David M. Gershenson, and  
Constantin G. Ioannides<sup>3</sup>

Departments of Gynecologic Oncology [B. W. A., D. M. G., C. G. I.], Surgery [G. E. P.], Bioimmunotherapy, [J. L. M., M. E. G.], and Immunology [C. G. I.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

## ABSTRACT

The presence of tumor-reactive CTLs in tumor infiltrates and in the peripheral blood of cancer patients demonstrates an immune response against tumors that apparently cannot control disease spread. This raises concerns as to whether amplification of this response may be useful during disease progression. Induction of tumor-reactive CTLs in healthy donors at risk, as well as in patients free of disease, may be therapeutically important, based on the hypothesis that CTLs that recognize tumors early may be more effective in containing their progression than CTLs that expand only when the disease progresses. To address the feasibility of priming cytolytic activity in healthy donors, we used the HER-2 peptide E75 (369–377) as an immunogen and autologous peripheral blood mononuclear cell-derived dendritic cells as antigen-presenting cells. We found that of 10 healthy donors tested, two responded at priming with E75 presented on autologous dendritic cells by induction of E75-specific CTL activity. Three other responders were identified after two additional restimulations. Of these five responders, three recognized E75 presented on the ovarian tumor line SKOV3.A2, as demonstrated by cold-target inhibition experiments. Induction of cytolytic activity at priming was enhanced in responders by tumor necrosis factor- $\alpha$  and interleukin 12 but not in the nonresponders.  $\alpha$ B7.1 monoclonal antibody added at priming enhanced induction of lytic activity in only one of the four nonresponding donors tested, suggesting that in the majority of donors, E75-precursor CTLs were not tolerized. Because of the possibil-

ity that disease may develop in nonresponders, strategies to improve the immunogenicity of tumor antigens for healthy donors may be required for development of cancer vaccines.

## INTRODUCTION

Identification of human epithelial tumor Ags,<sup>4</sup> such as the ones expressed on ovarian and breast cancers, allows antitumor vaccination strategies to be developed. Among the most interesting are those that focus on *HER-2* because this proto-oncogene is overexpressed in 20–40% of patients with highly aggressive breast, ovarian, pancreas, colon, and prostate cancers and with consequent poor prognosis. Two clinical trials have targeted *HER-2* (1, 2) using peptides and various adjuvants. The immunogen of choice in these trials was of the *HER-2* peptide E75 (369–377, KIFGSLAFL), which maps an epitope frequently recognized by CTLs from tumor-infiltrating/associated lymphocytes of breast and ovarian cancer patients (3, 4).

Although peptide immunization is an appealing approach to tumor immunotherapy because it removes concerns of toxicity and safety while focusing the effectors, the methodology for vaccination and immunological evaluation it is not yet defined (5). Important questions need to be addressed before this approach can be developed to its therapeutic potential. The first question is whether CTLs generated by primary *in vitro* and *in vivo* immunizations will lyse targets endogenously expressing the Ag. It has been shown with model Ag that the majority of peptide-induced CTLs at priming recognized the peptide used as immunogen, but only a small fraction recognized the endogenously presented Ag (6). In some instances, CTLs recognizing endogenously presented Ag could be induced only with a variant peptide (7). Although peptide-specific CD8<sup>+</sup> cells may not always be expected to directly lyse tumors *in vitro* and *in vivo*, such cells can recognize peptides derived from extracellular degradation of Ag from dying tumor cells and tumor debris. This may lead to secretion of various cytokine patterns in the tumor environment and conditioning of APCs, resulting in an indirect but significant impact on antitumor responses.

The second concern is how frequently tumor peptide vaccinations induce Ag-specific CTLs in the human population. This concern is attributable to the reported low precursor frequency of tumor-reactive CTLs in healthy individuals, however, this concept has been recently challenged (8, 9). There is also concern over the weak ability of tumor Ag to induce massive Ag-specific CTL expansions, as reported with viral Ags (10).

Received 4/17/00; revised 6/30/00; accepted 8/24/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported in part by Research Grant DAMD17-97-1-7098. Peptide synthesis was supported in part by Core Grant CA 16672.

<sup>2</sup> Present address: Department of Surgery, Uniformed Armed Services, University of the Health Sciences, Bethesda, MD 20814.

<sup>3</sup> To whom requests for reprints should be addressed, at The University of Texas M. D. Anderson Cancer Center, Department of Gynecologic Oncology, 1515 Holcombe Boulevard, Box 67, Houston, TX 77030. Phone: (713) 792-2849; Fax: (713) 792-7586.

<sup>4</sup> The abbreviations used are: Ag, antigen; DC, dendritic cell; *HER-2*, *HER-2/neu* proto-oncogene; HS, human serum; NP, no peptide; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; mAb, monoclonal antibody; TNF, tumor necrosis factor; LU, lytic unit(s); rVV, recombinant vaccinia vector.

The third concern is whether self-reactive (tumor-reactive) CTLs in healthy donors are silenced by active tolerance or anergy, and stimulation with Ag in peptide form cannot reactivate memory effectors because of B7-CTLA4-mediated peripheral tolerance. Recent studies have shown that induction of melanoma tumor Ag and tumor-reactive CTLs in healthy donors is much less effective than in cancer patients (10). However, induction of tumor-reactive CTLs in healthy donors (as well as in breast and ovarian cancer patients in long-term remission and without evidence of disease) is important based on the hypothesis that CTLs that recognize tumors early may be more effective in containing their progression than CTLs that expand only when the tumor Ag is overexpressed. (11).

Although a number of studies focused on improving the immunogenicity of tumor peptides in selected responding patients and donors using DCs as APCs and inflammatory cytokines, there is little information on the frequency of induction of these responses in unselected healthy donors. However, this question is important because cancer vaccines are expected to be given to distinct individuals, of which some may be at risk to develop disease, whereas others may be free of disease and otherwise considered healthy individuals. Thus, the frequency of CTL responses to a tumor Ag in the population becomes an important issue. We rationalized that if the frequency of responses to E75 priming is similar to or lower than the frequency of responses to MART-1 (10), initial screening of a large panel of healthy donors may identify at least one responder. Cells of this responder can be then used as positive controls to address the questions of costimulation and of cytokine help in elicitation of cytolytic function in nonresponders.

We developed a model for priming T cells of PBMCs from healthy donors with the HER-2 peptide E75. We used as APCs autologous DCs, always freshly generated in the presence of GM-CSF + IL-4 from the same PBMC sample. To determine the role of costimulation in this system,  $\alpha$ B7.1 antibodies were added at priming. To establish whether IL-12 and TNF- $\alpha$  are essential for CTL priming, stimulations were performed in the presence or absence of these cytokines. We found that 2 of 10 healthy donors responded by inducing E75-specific cytotoxicity at peptide priming and 5 of 10 at restimulation. Although IL-12 and TNF- $\alpha$  potentiated CTL induction in the responsive donors, they did not help induce CTLs in nonresponders, suggesting that additional factors to the nature of APCs and inflammatory cytokine conditioning regulate the induction of CTLs specific for HER-2 by synthetic peptides.

## MATERIALS AND METHODS

**Cells, Antibodies and Cytokines.** HLA-A2<sup>+</sup> PBMCs were obtained from healthy volunteers from the Blood Bank of M. D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study were: donor 1 (A2, B7, 44); donor 2 (A2, 33, B40, 44); donor 3 (A2, 33, B41, 81); donor 4 (A1, 2, B27, 44); donor 5 (A1, 2 B44, 57, Cw5, 6); and donor 6 (A2, 31, B35, 44, Cw4, w5). For the other four donors only, the HLA-A2 expression was determined. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (3, 4). mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and

B7.2 (CD80 and CD86, Calbiochem), intercellular adhesion molecule-1 (ICAM-1 CD54; Calbiochem), CD40L (Ansell, Bayport, MN), HLA-A2 (clone BB7.2; American Type Culture Collection), and MHC-II (L243; Dako Corp., Carpinteria, CA) were used as unconjugated, FITC, or phycoerythrin conjugated. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC; specific activity,  $1.25 \times 10^7$  colony-forming units/250 mg); TNF- $\alpha$  (Cetus Corp., Emeryville, CA; specific activity,  $2.25 \times 10^7$  units/mg); IL-4 (Biosource International; specific activity,  $2 \times 10^6$  units/mg); and IL-2 (Cetus Corp.; specific activity,  $18 \times 10^6$  IU/mg). IL-12 at  $5 \times 10^6$  units/mg was a kind gift from Dr. Stanley Wolf (Department of Immunology, Genetics Institute, Cambridge, MA).

**Synthetic Peptides.** The HER-2 peptides used were: E75 (369–377) and the unnatural modified Muc-1 peptides D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The corresponding natural peptides do not bind HLA-A2. Introduction of an HLA-A2 anchor and sequence modification in Muc1 in residues contacting TCR lead to an unnatural sequence (12). All peptides were prepared by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center and purified by high-performance liquid chromatography. Peptides were 95–97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at  $-20^\circ\text{C}$  in aliquots of 2 mg/ml Polyglycol bead-containing E75 were a kind gift of Dr. Kenneth Grabstein (Corixa Corp., Seattle, WA).

**Immunofluorescence.** Antigen expression by DCs and T cells was determined by fluorescence-activated cell sorter using a flow cytometer (EPICS Profile Analyzer; Coulter Co., Hialeah, FL). DCs were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DCs were incubated with phycoerythrin-conjugated anti-CD13 mAb and FITC-conjugated mAb specific for a surface Ag.

**Generation of PBMC-derived DCs.** CD13<sup>+</sup> DCs were generated from freshly isolated PBMCs by following the established CD14 methods (13, 14). Complete RPMI 1640 (containing 10% FCS) supplemented with 1000 units/ml GM-CSF, and 500 units/ml IL-4 were added to each well containing plastic-adherent cells, changed every 48 h, and maintained for 7 days. In separate studies, performed in parallel, we attempted to grow DCs in medium containing either HS or in AIM-V medium. The growth and expression of surface markers of DC cultured in complete RPMI 1640 was significantly better than of DCs cultured in other conditions; thus, in this study only DCs cultured in complete RPMI 1640 were used. CD8<sup>+</sup> cells were isolated by removing first the CD4<sup>+</sup> and then the CD16<sup>+</sup> and CD56<sup>+</sup> cells from the nonadherent population using Dynabeads (Dyna, Oslo, Norway). After depletion, the resulting cells were 97% CD8<sup>+</sup>, as determined by flow cytometry.

**T-Cell Stimulation by Peptide-pulsed DCs.** DCs were washed three times in serum-free medium, plated at  $1.2 \times 10^5$  cell/well in 24-well culture plates, and pulsed with peptides at 25–50  $\mu\text{g}/\text{ml}$  in serum-free medium for 4 h before the addition of responders. TNF- $\alpha$  (50 units/ml) was added to DCs for the last hour to stimulate Ag uptake and presentation (13). Autologous PBMCs or isolated CD8<sup>+</sup> cells in RPMI 1640 containing 10% HS were added to DCs at  $1.5 \times 10^6/\text{ml}$ , followed 60 min later by IL-12 at 3 IU/ml, IL-2 was added 16 h later to each well at 60 IU/ml and every 48 h thereafter. For inhibition studies,

Table 1 Summary of E75-specific cytolytic responses in healthy donors

Donor no.	E75-specific response at primary	Enhancement by IL-12/TNF- $\alpha$	E75-specific CTL at restimulation <sup>a</sup>	Specific tumor lysis	No. of independent priming experiments	
1	+	+	+	(1) <sup>b</sup>	+	6
2	-	-	-	(2)	ND	3
3	-	-	+	(3) <sup>c</sup>	+	6
4	-	-	-	(3)	ND	4
5	+	+	+	(1)	+	4
6	-	-	+	(3) <sup>c</sup>	-	5
7	$\pm$ <sup>d</sup>	$\pm$	+	(1)	-	4
8	-	-	-	(2)	ND	4
9	- <sup>e</sup>	-	-	(2)	ND	3
10	-	-	-	(1)	ND	1
Total	2/10	2/10	5/10	3/5	40	

<sup>a</sup> +, specific lysis of E75-pulsed T2 was  $\geq 20\%$  at an E:T ratio of 20:1; -, specific lysis of E75-pulsed T2 was not significantly different than lysis of control T2 cells pulsed with no peptide. ND, not done.

<sup>b</sup> Numbers in parentheses, number of restimulations.

<sup>c</sup> E75-specific CTL activity was detected after four stimulations.

<sup>d</sup> E75-specific CTL activity was  $\leq 10\%$  at an E:T of 10:1.

<sup>e</sup> E75-specific activity, observed occasionally, could not be enhanced by peptide plus cytokine stimulation but was enhanced by  $\alpha B7.1$ .

mAbs specific for B7.1, B7.2, and HLA-A2 were added to DCs 1 h before responders in amounts reported to be inhibitory by the manufacturers.

**CTL and Cytokine Assays.** Recognition of peptides used as immunogens by CTLs was performed as described (3, 15). Equal numbers of viable effectors from each well were used in all assays. To minimize cross-reactive recognition of human peptides, all stimulations were performed in medium containing HS, whereas CTL assays were performed using medium containing FCS (15). This minimized background cytotoxicity attributable to activation of other autoreactive cells to Ags present in HS. Specific LU were determined as described (10, 16) and are expressed as LU  $30/10^7$  cells.

## RESULTS

**Priming of Healthy Donors PBMCs with E75-pulsed Autologous CD14-derived DCs.** To address the question of whether priming with E75 presented on DCs induce E75-specific CTL activity, plastic nonadherent PBMCs from healthy donors were stimulated with autologous DCs generated by culture in GM-CSF + IL-4. DCs were pulsed with E75 at 25–50  $\mu\text{g/ml}$ . Seven days later, cytolytic activity was determined against E75-pulsed T2 using as control T2 cells that were not pulsed with peptide. Cumulative results are presented in Table 1. The results show that of 10 healthy donors tested, only 2 showed stable and consistent recognition of E75 after priming with DC-E75. These cumulative results show that E75 can induce specific cytolytic activity at priming only in a small fraction of healthy donors (2 of 10; 20%). These results were confirmed because each CTL induction experiment was repeated at least three times at different time points and always with freshly isolated PBMCs (except donor 10). In 2 donors (donors 2 and 9), specific CTL activity was occasionally de-

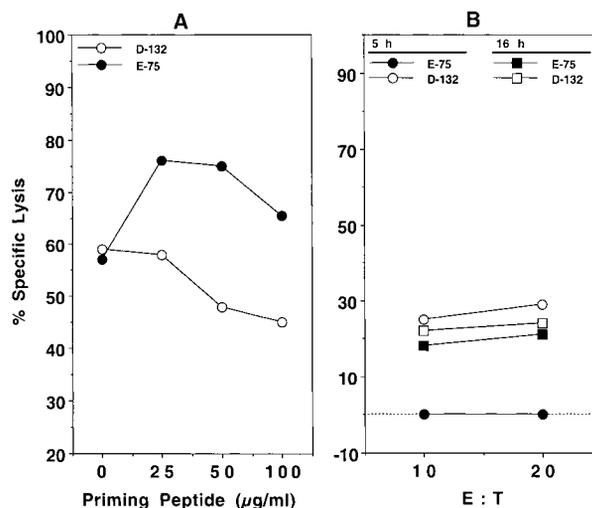
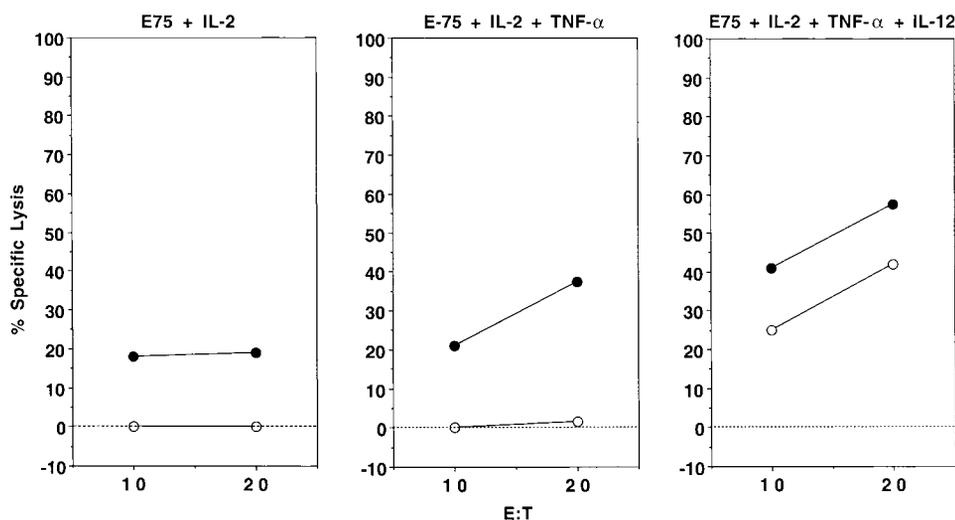


Fig. 1 A, induction of E75-specific cytolytic activity at priming with various concentrations of E75. Plastic nonadherent PBMCs from donor 1 were stimulated with the indicated concentrations of E75. Seven days later, cells were removed from cultures and tested for CTL activity against T2 pulsed with 25  $\mu\text{g/ml}$  E75 or D132. The experiment was performed in triplicate at a 20:1 ratio from all stimulation groups. Differences between T2-E75 (●) recognition and T2-D132 recognition (○) are significant ( $P < 0.05$ ) in all DC-E75 stimulation groups. B, priming of PBMCs from donor 9 (nonresponder) with E75 in polyglycol beads does not induce E75-specific CTL activity. Experimental conditions were as described for donor 1.

ected in one of three independent experiments, but this activity was unstable and could not be further expanded. Induction of this activity in donor 9 required  $\alpha B7.1$  at priming (shown in Fig. 5). In contrast, in the two responding donors, specific CTL activity was detected at priming in 4 of 6 (donor 1) and 3 of 4 (donor 5) independently performed induction experiments over periods of 1 year and 6 months, respectively.

To address the question of the optimal Ag concentration for priming, autologous DCs were pulsed with 0, 25, 50, and 100  $\mu\text{g/ml}$  of E75 prior to the addition of responders. The resulting cultures were supplemented with TNF- $\alpha$  and IL-12 at priming and IL-2 every 48 h for the following 7 days. A representative experiment of the specificity of recognition of CTLs from donor 1 primed by DC-E75 is shown in Fig. 1. The results show that the specificity of recognition of CTLs induced by E75 at three concentrations of peptide (25, 50, and 100  $\mu\text{g/ml}$ ) was similar, with 50  $\mu\text{g/ml}$  E75 inducing the highest differences in lytic activity between targets pulsed with E75 or targets pulsed with no peptide. CTLs could not be induced even by high concentrations of E75 in any of the nonresponders. To address whether E75-stimulatory potency was increased by delivery of Ag encapsulated in polyglycol beads, PBMCs from two nonresponders (numbers 2 and 9) were stimulated with autologous DCs pulsed with polyglycol beads in numbers to generate equivalent E75 concentrations with peptides. The results are shown for donor 9 (Fig. 1B). Stimulation with encapsulated-E75 failed to induce specific CTL activity at priming, as concluded from 5-h CTL assays. However, in longer CTL assays (16 h), lysis of T2-E75 pulsed targets increased significantly compared with

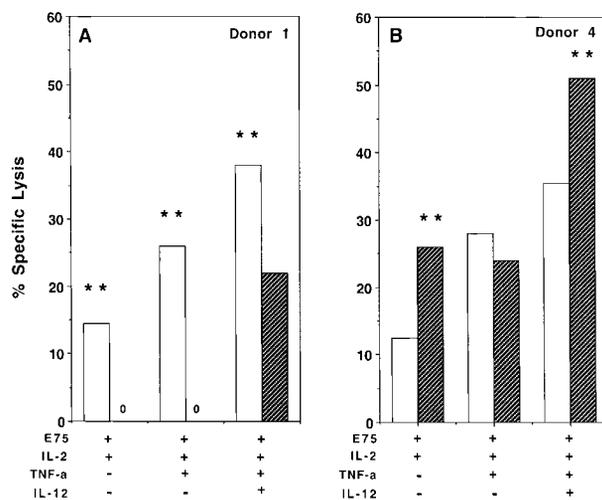
**Fig. 2** Induction of E75-specific cytolytic activity at priming in donor 5. Plastic non-adherent PBMCs were stimulated with 50  $\mu\text{g/ml}$  E75 pulsed on autologous DCs.  $\text{TNF-}\alpha$  (50 IU/ml), IL-12 (3 IU/ml), and IL-2 (60 IU/ml) were added to these cultures. Differences in lysis of T2 cells pulsed with E75 (●) or not (○) are significant in all groups ( $P < 0.02$ ). All experiments were performed simultaneously in triplicate. Results of one experiment of two performed with similar results are shown.



that of control targets, and the levels of recognition were similar. This suggested that either the numbers of E75-specific CTLs were low, or CTLs in these two donors had weak cytolytic activity. It should be mentioned that in both experiments, IL-2 was added at 48 and 96 h in higher concentrations (150–200 IU/ml) to facilitate T-cell expansion. Although this increased the background lysis, it did not change the patterns of recognition.

**Effects of  $\text{TNF-}\alpha$  and IL-12 in Induction of CTL Activity at Priming.** Both  $\text{TNF-}\alpha$  and IL-12 have been described in different systems to augment cytotoxicity of  $\text{CD8}^+$  cells (17, 18). To determine whether the E75-induced CTL activity could be enhanced by  $\text{TNF-}\alpha$  and IL-12, CTL priming experiments were performed in the presence and absence of these cytokines (Fig. 2). The results from donor 5 show that the addition of  $\text{TNF-}\alpha$  at the time of pulsing with peptide-enhanced, E75-specific activity compared with cultures that received only IL-2. When IL-12 was added, the increase in T2-E75 killing was paralleled by an increase in nonspecific killing. The addition of IL-12 at higher concentrations during priming did not increase the specific but rather the nonspecific CTL activity. This was equally true when isolated  $\text{CD8}^+$  cells were used as effectors (data not shown). To address whether these cytokines enhanced cytolytic activity at priming in nonresponders, the experiment was repeated with donor 1 (responder) and donor 4 (non-responder). The results in Fig. 3 show that  $\text{TNF-}\alpha$  increased specific CTL activity in donor 1 but not in donor 4. Although T2-E75 lysis increased in  $\text{TNF-}\alpha$ -treated cultures, it was still not significantly different from the control. Similar to donor 5 (in both donors 1 and 4), IL-12 increased both the nonspecific and specific lysis. These results were confirmed with all donors tested (Table 1). IL-12 alone, or together with IL-2 and  $\text{TNF-}\alpha$ , failed to induce specific CTL activity in nonresponders.

To address whether the increase in cytolytic activity induced by  $\text{TNF-}\alpha$  and IL-12 at priming was attributable to changes in the levels of  $\text{CD8}^+$  cells in these cultures, we determined the percentages of  $\text{CD8}^+$  and  $\text{CD4}^+$  in E75-primed cultures from donor 5 used in the experiment shown in Fig. 2. The results in Table 2 show only small differences between the



**Fig. 3** Addition of IL-12 at priming does not induce specific CTL activity in the nonresponders. Donor 1 (responder; A) and donor 4 (nonresponder; B) were tested in the same experiment. The E:T ratio was 20:1. For donor 1, this experiment was performed at a different date with a fresh blood sample than the experiment shown in Fig. 1. A: \*\*, significant differences in recognition of E75 (□) compared with control NP (■);  $P < 0.02$ . B: \*\*, significantly higher recognition of T2-NP than of T2-E75 ( $P < 0.05$ ).

percentage of  $\text{CD8}^+$  cells in cultures containing IL-2 only compared with IL-2 +  $\text{TNF-}\alpha$  or IL-2 +  $\text{TNF-}\alpha$  + IL-12. A caveat of this analysis is that E75 tetramers are not yet available; thus, we could not determine whether  $\text{TNF-}\alpha$  and IL-12 increased the numbers of E75-specific cells in these cultures.

**Involvement of B7-CD28 Costimulation in E75-specific CTL Priming.** To address whether induction of E75-specific cytolytic activity required costimulation,  $\alpha\text{B7.1}$  and  $\alpha\text{B7.2}$  mAbs were added at priming. The results in Fig. 4 show that  $\alpha\text{B7.1}$  significantly inhibited induction of specific CTL activity in donor 5 (by >80%), whereas  $\alpha\text{B7.2}$  had a much smaller effect.

Table 2 Expression of CD8 and CD4 on E75-primed cultures

Peptide	IL-2	TNF- $\alpha$	IL-12	%CD8 <sup>+</sup>	%CD4
-	+	-	-	21.3	59.1
+	+	-	-	18.9	64.9
-	+	+	-	N.D.	N.D.
+	+	+	-	21.4	63.4
-	+	+	+	25.2	55.9
+	+	+	+	25.1	55.0

Plastic-nonadherent PBMCs from donor 5 were stimulated with autologous DCs pulsed with E75, or as control with autologous DCs that were not pulsed with E75. TNF- $\alpha$  and IL-12 were added at priming, whereas IL-2 was added 24 h later. Cells were stained with the corresponding  $\alpha$ -CD8 and  $\alpha$ -CD4 in antibody 7 days later and examined by fluorescence-activated cell sorter.

B7.2 was expressed at significantly higher levels than B7.1 on DCs. Furthermore, interaction of DCs with T cells and cytokines was paralleled by B7.2 but not B7.1 up-regulation within 20 h (not shown), raising the possibility that  $\alpha$ B7.2 mAb was insufficient for blocking. The strong inhibition of E75-specific CTL induction by  $\alpha$ B7.1 suggested that the responder CTLs in this donor are more likely naïve T cells.

To determine whether the nonresponders were activated but tolerized T cells, which cannot expand because of B7-CTLA4 interaction, the experiment was repeated with four nonresponders (nos. 3- 6) using the same amounts of  $\alpha$ B7.1 as in donor 5. The results are shown in Fig. 5. In donor 9, the addition of  $\alpha$ B7.1 at priming led to induction of specific CTL activity (Fig. 5A). It is tempting to speculate that in this donor, activated but tolerized E75-specific CTLs were present, and they cannot expand because of the B7-CTLA4 interaction. Additional studies are needed to address this point. In donors 4 and 5, the addition of  $\alpha$ B7.1 at priming failed to induce significant specific cytolytic activity. These results were confirmed with donor 3 (not shown). Thus, of five donors tested,  $\alpha$ B7.1 inhibited E75-specific CTL priming in one (no. 5), enhanced CTL priming in another one (no. 9), but failed to enhance induction of specific CTL activity in three (nos. 3-5). These experiments were repeated, and the results were confirmed. Thus, the requirements for B7-CD28 costimulation appeared to be dependent on the donor.

**Induction of CTL Activity at Restimulation.** To address whether E75 restimulation enhanced specific cytolytic activity, E75 primed PBMCs from all donors were restimulated with DC-E75. Of the eight nonresponders at primary stimulation, only three (donor nos. 3, 6, and 7) increased their E75-specific lysis at restimulation. In two of three donors (donors 3 and 6), specific E75 recognition was borderline after restimulation. E75-specific cytotoxicity was observed at the fourth stimulation with DC-E75 in these two donors (data not shown). In the other five donors that were both primed and restimulated with E75 but failed to show specific CTL activity, additional restimulations were not attempted, because of the low levels of recognition of T2-E75 at restimulation compared with nonspecific lysis. We rationalized that the five nonresponders will require a minimum of four and even more restimulations to possibly elicit E75-specific CTLs. Thus, even if cytolytic activity would be detected after four to five stimulations, this finding would also support the hypothesis of weak E75 immunogenicity in these individuals.

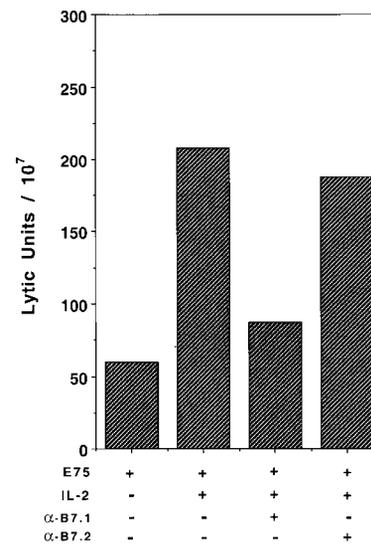
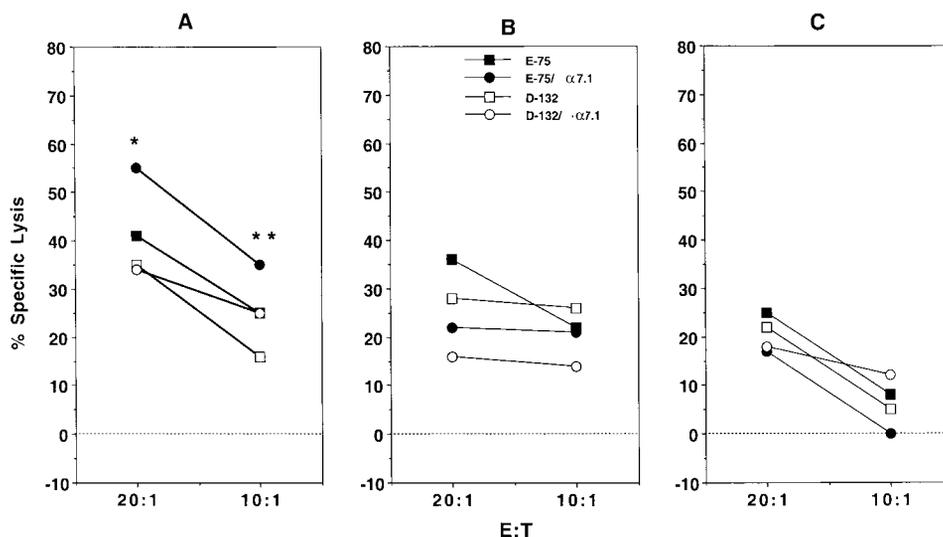
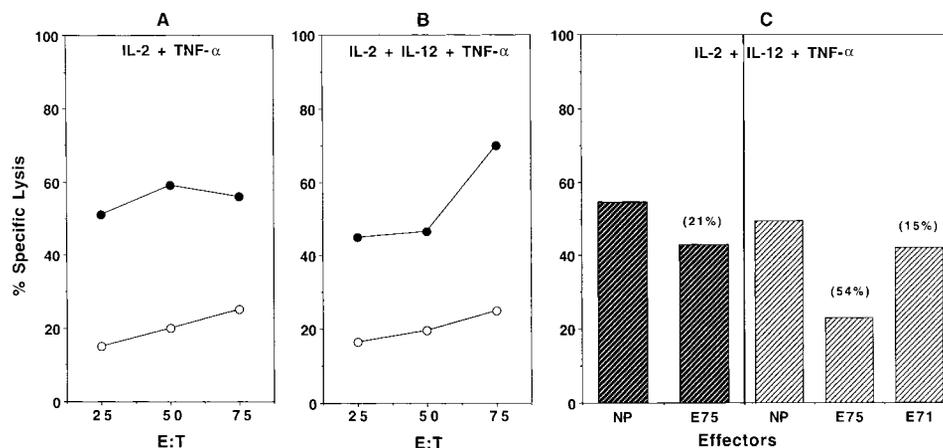


Fig. 4 Priming of E75-specific cytotoxicity in donor 5 requires B7 costimulation. Plastic nonadherent PBMCs from donor 5 were primed with E75 in the presence or absence of  $\alpha$ B7.1 and  $\alpha$ B7.2 mAb. Results are shown as specific LU calculated from the percentage of specific lysis against E75 and against the specificity control, the unnatural negative control peptide D132 (10, 12) at two E:T ratios (10:1 and 20:1). D132 was used as control to stabilize HLA-A2 on T2 cells similarly with E75.

**Recognition of Tumor Cells by E75-primed CTLs.** To address the question of whether E75-primed CTLs from healthy donors recognized endogenously presented epitopes, CTLs generated from donors 1, 3, 5, 6, and 7, which showed peptide-specific lytic activity, were tested for their ability to lyse the tumor line SKOV3.A2 and its A2<sup>-</sup> counterpart, SKOV3. Except for HLA-A2, all other histocompatibility Ags on SKOV3 and SKOV3.A2 are identical. To verify that the responses are E75 specific, cold-target inhibition experiments using unlabeled T2-E75 as specific target and T2-NP as negative control were performed in parallel. T2 express only HLA-A2 and low levels of HLA-B5. The results are summarized in Table 1. Peptide-specific CTLs from donors 6 and 7 did not show specific recognition of SKOV3.A2 tumor. However, E75-specific CTLs from donors 1, 3, and 5 recognized endogenous E75. These donors do not express HLA-B5, suggesting that E75 was presented by HLA-A2. It should be mentioned that E75-specific CTLs were induced in donors 1 and 5 at priming with DC-E75, whereas in donor 3, expression of this cytolytic activity required four stimulations with DC-E75. These results indicated that of 10 healthy donors tested, only three (33%) responded by inducing CTLs that specifically recognized tumor cells. This percentage was higher in the group of responders with peptide-specific CTLs (three of five; 60%). The results with donor 5 are shown in Fig. 6, A and B. Both E75-primed cultures from donor 5 lysed SKOV3.A2 better than SKOV3, suggesting that they recognize an epitope associated with HLA-A2. To address whether these cultures recognized an endogenous presented epitope similar to E75, we performed cold-target inhibition experiments. The results in Fig. 6C show that T2-E75 significantly inhibited by >50% recognition of SKOV3.A2 by CTLs from donor 5 com-



**Fig. 5** Effects of B7.1 blocking on priming of E75-peptide-specific cytolytic activity in distinct healthy donors. Induction of E75-specific cytotoxicity by blocking of B7.1 in donor 9 (A) but not in donors 3 and 4 (B and C).  $\alpha$ B7.1 was added to DCs 30 min before the addition of responders. Cultures were primed with E75 in the presence (●) or absence (□, ■) of  $\alpha$ B7.1 in the same experiment. Cytolytic activity was determined in the same experiment in triplicate against targets pulsed with E75 (■, ●) or the control unnatural peptide D132 (□, ○). D132 was used to stabilize HLA-A2 at comparable levels with E75, thus minimizing the natural killer-derived background lysis. \* and \*\*,  $P < 0.01$  and  $< 0.05$  respectively, compared with the lysis of T2-D132.



**Fig. 6** A and B, E75-primed PBMCs from donor 5 recognize SKOV3.A2 (●) better than SKOV3 (○) cells, suggesting that they recognize an HLA-A2-associated Ag. Differences between the levels of lysis of two targets are significant ( $P < 0.05$ ). C, cold-targeted inhibition of SKOV3.A2 lysis by E75 + IL-2 + TNF- $\alpha$ -induced cells by T2-E75, but not by negative control T2-NP, or the negative peptide control T2-E71 demonstrate E75-specific recognition for donor 5 (■) and donor 1 (□). Differences in lysis of SKOV3.A2 cells incubated with T2-NP (percentage of specific lysis: 56, 59, and 60%) and T2-E75 (percentage of specific lysis: 39, 42, and 44%) were significant ( $P < 0.05$ ).

pared with control T2-NP, which expressed HLA-A2. This inhibition was peptide specific because it was not observed with the control peptide E71 pulsed on T2, suggesting that some E75-primed CTLs recognized an endogenously presented epitope, but these cells are not the majority in the effector population. Similar results were obtained with E75-primed cells from donors 1 (Fig. 6C) and 3 (not shown). However, the levels of cold-target inhibition were lower and ranged between 20 and 25% in two separate experiments. This suggested that only a subpopulation of E75-induced CTLs recognize endogenous gen-

erated epitopes. Thus, successful induction of E75-specific CTL activity at priming with E75 using DCs as APCs and inflammatory cytokine support appears to be dependent of additional factors other than the nature of APCs and B7 costimulation.

## DISCUSSION

In this study, we investigated the ability of the HER-2 peptide E75 to prime E75-specific cytolytic activity in healthy donors when presented on autologous DCs. We found that only

2 of the 10 HLA-A2<sup>+</sup> healthy donors tested responded by induction of E75-specific cytolytic activity at priming. This was confirmed in replicated experiments performed over time, and the use of various cytokine combinations IL-2+IL-12, IL-2 + TNF- $\alpha$  + IL-12, or preculture in IL-2, preculture in IL-2 + RANTES.<sup>5</sup> These results indicated that E75-specific or cross-reactive T cells endowed with cytolytic activity can be elicited at priming in only a fraction of healthy donors (20%) but induced in an additional 30%. Of interest, E75-primed CTLs from these two donors recognized E75 presented on tumor cells, because their activity was inhibited in cold-target inhibition assays.

Two cytokines, TNF- $\alpha$  and IL-12, were used to potentiate E75-specific CTL induction. TNF- $\alpha$  has been described to increase Ag uptake and presentation by DCs (13) and to potentiate CTL generation in animal models (17). IL-12 has also been described to potentiate CTL induction and cytolytic activity (18, 19). TNF- $\alpha$  and IL-12 increased the levels of cytolytic activity in responders but had no effect in nonresponders. This suggests that these cytokines are not essential for priming of E75-specific CTL activity. Of interest, in the responders, induction of E75-specific cytolytic activity was inhibited by  $\alpha$ B7.1, suggesting a requirement for costimulation in the induction of cytolytic activity as described (20) for other Ag raising the possibility of DC-E75 primed naïve T cells.

Induction of E75-specific cytolytic activity at priming with peptide observed with two healthy donors is of interest in evaluating the potential of this epitope for tumor-specific CTL induction and cancer vaccine development. In human tumor systems (most instances), priming with peptide required several repeated stimulations of healthy donor PBMCs before specific cytotoxicity was detected. CTLs specific for tyrosinase 369–377 peptide were detected in four of five healthy donors after three restimulations with peptide (21). Peptide-specific CTLs were induced in healthy donors using DCs and peptides from gp100, tyrosinase, and MART-1/MelanA. Detection of CTL activity required three to four cycles of stimulation (22). Similarly, presentation of MART-1 by DCs transduced with an adenoviral vector construct carrying the *MART-1* gene required three stimulations for induction of specific cytotoxicity (23), although in some donors, specific cytolytic effectors were detectable 7 days after priming (24). In contrast, in another study, MART-1 (27–35)-specific cytolytic function could be induced in a nonresponder only, using APCs infected with rVVs expressing rVV-B7.1/2 + peptide, or rVV-B7.1 + MART-1 and restimulated with peptide, but not by peptide stimulation only (25, 26). Thus, the potency of E75 to induce cytolytic function in healthy donors appears similar to that of the MART-1 peptide 27–35.

Few studies have investigated the frequency of responses to tumor Ags in healthy donors at priming and restimulation or the consistency of these responses for an individual. This aspect is important because of its implications for protective vaccination in healthy donors or ovarian, breast, and prostate cancer patients without evidence of disease. In one extensive study, Marincola *et al.* (10) found that after several stimulations with

MART-1 (27–35), five of nine healthy donors (56%) responded by induction of specific cytolytic effectors. Only one to two donors showed weak activation of cytotoxicity at priming. In an independent study, 4 of 16 healthy donors (25%) responded to MART-1 (27–35) after two stimulations (27). Similarly, anti-p53 (264–272) cytolytic effectors were generated from 2 of 5 healthy donors (40%) after several restimulations with peptide-pulsed DCs (28), whereas anti-gp100 CTLs were elicited in 1 of 10 healthy donors at priming (29).

In contrast with melanoma Ag, the immunogenicity of which has been repeatedly investigated in extensive studies, the experience with E75 is limited. Similar to the MART-1 peptide, E75 activated rapid cytokine secretion from cultured ovarian tumor-infiltrating lymphocytes or CTL lines (30, 31) and activated cytotoxicity in tumor-associated lymphocytes (32). Freshly isolated PBMCs from cancer patients that were not vaccinated with peptide rapidly responded to E75 and to another HER-2-epitope, GP2 (33), in a similar fashion as melanoma patients to MART-1, by induction of specific cytotoxicity and tumor recognition (34). The ease by which E75- and GP2-specific cytolytic activities were induced in patients suggested that E75 and GP2 reactivated effector/memory CTLs rather than primed naïve cells (34). Our results showing 2 of 10 responders at priming (20%) and 5 of 10 responders at restimulation (50%) indicate that E75 is similar to MART-1 (27–35), tyrosinase (369–377), and p53 (264–272) in its ability to activate cytotoxicity in randomly selected healthy donors.

It is possible that E75 cannot elicit a complete response in all donors during PBMC priming. Our recent studies on cytokine responses by E75-primed PBMCs in healthy donors show that E75 rapidly activated specific IFN- $\gamma$  release in five of six healthy donors, an effect that was enhanced by IL-12 (36). Although the same donors were used in these studies and IL-12 was used in parallel experiments, we could not observe a similar effect with respect to induction of cytotoxicity. Thus, a complete response (cytokines and cytotoxicity) was observed only in two donors of the six tested. This suggests that E75 may act as a partial agonist. In support of this possibility, Zaks and Rosenberg (1) reported recently that E75 vaccination in incomplete Freund adjuvant of four cancer patients led to a peptide-specific response at restimulation in all patients (cytotoxicity and IFN- $\gamma$ ). T cells from two of three E75-vaccinated patients recognized, occasionally, tumor cells by specific IFN- $\gamma$  secretion but failed to show specific tumor lysis (1). Similarly, tyrosinase 369–377-specific CTLs from two of four responders failed to recognize tyrosinase-expressing tumors (21). Preliminary results from a vaccine trial in breast and ovarian cancer patients indicate that PBMCs from only two of six E75-vaccinated patients (33%) stimulated *in vitro* elicited specific CTL activity against peptide and specific tumor recognition, although all responded to E75 by specific IFN- $\gamma$  induction (2).

The similar response rates for induction of cytotoxicity in healthy donors to *in vitro* tumor peptide vaccination raise a number of questions about the application of this approach:

(a) Why, regardless of the Ag used, only 10–20% of healthy donors respond at priming, and only 40–50% at restimulation with peptide? One possibility is that healthy donors have different precursor frequencies for the CTL epitopes, and repeated peptide stimulation (three to four times) is not sufficient to expand the

<sup>5</sup> T. V. Lee and C. G. Ioannides, preliminary data.

effector population to sufficiently high numbers to detect CTL activity. Thus, one approach is to continue repeated vaccinations and deliver exogenous help by helper peptides and cytokines until such CTL responses are elicited (9, 35).

(b) If the 10–20% of donors that respond to peptide priming have higher pCTL frequencies for E75/MART-1 than nonresponders, then what is the reason for this increased frequency? It is tempting to speculate that local inflammatory conditions and cross-reactive priming may activate CTL precursors, such as in donor 9, and these precursors become tolerized.

(c) If pCTL frequency is similarly low in all healthy donors, then why do some respond better than others? One possibility is that discrete changes in HLA-A2 attributable to HLA-A2 polymorphism may lead to a more immunogenic E75 in some donors. In support of this possibility, Maurer *et al.* (37) demonstrated that mutated HLA-A2 in position 97 can segregate MART-1 (27–35)-induced cytolysis from cytokine production. Furthermore, the pool of E75 precursors may expand or contract over time because of different environmental factors (38, 39). This may be supported by the fact that the responders showed E75-specific CTL activity frequently, whereas some of the nonresponders showed activity only occasionally in the majority of independently performed experiments. Additional studies using carboxyfluorescein acetate to determine cell division, E75-tetramers to determine the frequency of E75-specific CTLs, and intracellular IFN- $\gamma$  staining are required to distinguish among these possibilities.

Increased HLA-A2 binding affinity by COOH-terminal modification was able to enhance tumor Ag immunogenicity (in some instances), as shown in our previous studies (15) and by other investigators using the melanoma Ag gp100 (40, 41). However, increased HLA-A2 binding affinity does not always predict a higher T-cell receptor signaling or a complete T-cell activation (reviewed in Ref. 42). In some of the reported cases, CTLs induced by higher HLA-A2 affinity binding variant showed low affinity for the tumor cells (15), and more recently (40, 41), some reports have suggested that they preferentially targeted tumors expressing high numbers of the epitope. Thus, novel immunogens need to be designed with emphasis on modifications in the Ag that would induce high rates of proliferation and select responders of high cytolytic activity, *i.e.*, high catalytic activity as demonstrated by enzymes. Because restimulations may induce apoptosis, it remains to be seen whether tumor-specific CTL expansion would require several agonists, each being capable of activating one effector function at a time. At the present, the results of this study demonstrated that cytolytic effectors to an epitope on HER-2, which is overexpressed on the majority of epithelial tumors, can be elicited in a fraction of healthy individuals at priming, and in nonresponders, the precursors were not tolerized. Because these studies were performed with 10 donors and substantiated in multiple replicate induction experiments using the same stimulation system, this suggests that this stimulation system may be identifying individuals that will respond to vaccine with a tumor Ag. This may have implications for preventative vaccination in high-risk individuals.

## ACKNOWLEDGMENTS

We thank the Blood Bank personnel for donating blood repeatedly for this project.

## REFERENCES

- Zaks, T. Z., Rosenberg, S. A. Immunization with a peptide epitope (p369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu<sup>+</sup> tumors. *Cancer Res.*, 58: 4902–4908, 1998.
- Anderson, B., Lu, J. J., Ibrahim, N., Hortobagyi, G., Brewer, H., Przepiorka, D., Ioannides, C., Grabstein, K., Cheever, M., and Murray, J. Immunomodulating effects of a HER2/neu derived HLA-A2 specific peptide E75 combined with GM-CSF in patients with metastatic breast and ovarian cancer. *Proc. Am. Soc. Clin. Oncol.*, 18: A1691, 1999.
- Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. 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.
- Hudson, J. M., Castilleja, A., Murray, J. L., Honda, T., Kudelka, A., Singletary, E., Wharton, J. T., and Ioannides, C. G. Growth and antigen recognition of tumor-infiltrating lymphocytes from human breast cancer. *J. Interferon Cytokine Res.*, 18: 529–536, 1998.
- Dudley, M. E., Nishimura, M., I., Holt, A. K. C., and Rosenberg, S. A. Antitumor immunization with a minimal peptide epitope (G9–209-2M) leads to a functionally heterogeneous CTL response. *J. Immunother.*, 22: 288–298, 1999.
- Carbone, E. R., Moore, M. W., Sheil, J. M., and Bevan, M. J. Induction of cytotoxic T lymphocytes by primary *in vivo* stimulation with peptides. *J. Exp. Med.*, 167: 1767–1779, 1988.
- Kelly, J. M., Sterry, S. J., Cose, S., Turner, S. J., Fecondo, J., Rodda, S., Fink, P. J., and Carbone, F. R. Identification of conserved T cell receptor CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. *Eur. J. Immunol.*, 23: 3318–3326, 1993.
- Pittet, M., Valmori, D., Dunbar, P. R., Speiser, D. E., Lienard, D., Lejeune, F., Fleischhauer, K., Cerundolo, V., Cerottini, J.-C., and Romero, P. High frequencies of naïve melan-A/MART-1-specific CD8<sup>+</sup> T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2-individuals. *J. Exp. Med.*, 190: 705–715, 1999.
- Anichini, A., Molla, A., Mortarini, R., Tragni, G., Bersani, I., DiNicola, M., Gianni, A. M., Piloti, S., Dunbar, R., Cerundolo, V., and Parmiani, G. An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. *J. Exp. Med.*, 190: 651–667, 1999.
- Marincola, F. M., Rivoltini, L., Salgaller, M. L., Player, M., and Rosenberg, S. A. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of *in vivo* priming by tumor cells. *J. Immunother.*, 19: 266–277, 1996.
- Alexander-Miller, M. A., Leggatt, G. R., and Berzofsky, J. A. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc. Natl. Acad. Sci.*, 93: 4102–4107, 1996.
- Ioannides, C. G., Fisk, B., Jerome, K. R., Chesak, B., Irimura, T., Wharton, J. T., and Finn, O. J. Ovarian tumors reacting cytotoxic T lymphocytes can recognize peptide determinants on polymorphic epithelial mucins. *In: F. Sharp, P. Mason, J. Berek, and A. Blackett (eds.), Ovarian Cancer*, pp. 317–330. London: Chapman and Hall, 1994.
- Sallusto, F., and Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor. *J. Med. Exp.*, 179: 1109–1118, 1994.
- Pickl, W. F., Majdic, O., Kohl, P., Stockl, J., Riedl, E., Scheinecker, C., Bello-Fernandez, C., and Knapp, W. Molecular and functional characteristics of dendritic cells generated from highly purified CD14<sup>+</sup> peripheral blood monocytes. *J. Immunol.*, 157: 3850–3859, 1996.
- Fisk, B., Chessak, B., Pollack, M., Wharton, J. T., and Ioannides, C. G. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene *in vitro*. *Cell. Immunol.*, 157: 412–427, 1994.

16. Ioannides, C. G., Platsoucas, C. D., Rashed, S., Wharton, J. T., Edwards, C. L., and Freedman, R. S. Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. *Cancer Res.*, *51*: 4257–4265, 1991.
17. Gorelik, L., Bar-Dagan, Y., and Mokyr, M. B. Insight into the mechanism(s) through which TNF promotes the generation of T cell-mediated antitumor cytotoxicity by tumor bearer splenic cells. *J. Immunol.*, *156*: 4298–4308, 1996.
18. Mehrotra, P. T., Wu, D., Crim, J. A., Mostowski, H. S., and Siegel, J. P. Effects of IL-12 on the generation of cytotoxic activity in human CD8<sup>+</sup> T lymphocytes. *J. Immunol.*, *151*: 2444–2452, 1993.
19. Bhardwaj, N., Seder, R. A., Reddy, A., and Feldman, M. V. IL-12 in conjunction with dendritic cells enhances antiviral CD8<sup>+</sup> CTL responses *in vitro*. *J. Clin. Investig.*, *98*: 715–722, 1996.
20. Guerder, S., Carding, S. R., and Flavell, R. A. B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. *J. Immunol.*, *155*: 5167–5174, 1995.
21. Visseren, M. J. W., van Elsas, A., van der Voort, E. I. H., Rensing, M. E., Kast, W. M., Schrier, P. I., and Melief, J. M. ACTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. *J. Immunol.*, *154*: 3991–3998, 1995.
22. Bakker, A. B. H., Marland, G., de Boer, A. J., Huijbens, R. J. F., Danen, E. H. J., Adema, G. J., and Figdor, C. G. Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells *in vivo*. *Cancer Res.*, *55*: 5330–5334, 1995.
23. Perez-Diez, A., Butterfield, L. H., Li, L., Chakraborty, N. G., Economou, J. S., and Mukherji, B. Generation of CD8<sup>+</sup> and CD4<sup>+</sup> T-cell response to dendritic cells genetically engineered to express the *MART-1/Melan-A* gene. *Cancer Res.*, *58*: 5305–5309, 1998.
24. Butterfield, L. H., Jilani, S. M., Chakraborty, N. G., Bui, L. A., Ribas, A., Dissette, V. B., Lau, R., Gamradt, S. C., Glaspy, J. A., McBride, W. H., Mukherji, B., and Economou, J. S. Generation of melanoma-specific cytotoxic T lymphocytes by dendritic cells transduced with a MART-1 adenovirus. *J. Immunol.*, *161*: 5607–5613, 1998.
25. Zajac, P., Oertli, D., Spagnoli, G. C., Noppen, C., Schaefer, C., Heberer, M., and Marti, W. R. Generation of tumoricidal cytotoxic T lymphocytes from healthy donors after in stimulation with a replication into competent vaccinia virus encoding MART-1/Melan-A 27–35 epitope. *Int. J. Cancer*, *71*: 491–496, 1997.
26. Zajac, P., Schutz, A., Oertli, D., Noppen, C., Schaefer, C., Heberer, M., Spagnoli, G. C., and Marti, W. R. Enhanced generation of cytotoxic T lymphocytes using recombinant vaccinia virus expressing human tumor-associated antigens and B7 costimulatory molecules. *Cancer Res.*, *58*: 4567–4571, 1998.
27. Chen, Q., Jackson, H., Gibbs, P., Davis, I. D., Trapani, J., and Cebon, J. Spontaneous T cell responses to melanoma differentiation antigens from melanoma patients and healthy subjects. *Cancer Immunol. Immunother.*, *47*: 191–197, 1998.
28. Chikamatsu, K., Nakano, K., Storkus, W. J., Appella, E., Lotze, M. T., Whiteside, T. L., and DeLeo, A. B. Generation of anti-p53 cytotoxic T lymphocytes from human peripheral blood using autologous dendritic cells. *Clin. Cancer Res.*, *5*: 1281–1288, 1999.
29. Jager, E., Ringhoffer, M., Arand, M., Karbach, J., Jager, D., Ilse-mann, C., Hagedorn, M., Oesch, F., and Knuth, A. Cytolytic T cell reactivity against melanoma-associated differentiation antigens in peripheral blood of melanoma patients and healthy individuals. *Melanoma Res.*, *6*: 419–425, 1996.
30. Wasserman, K., Corsi, M. M., Szekely, L., Kono, K., Maes, H. H., and Kiessling, R. Conversion of *in vitro* cultured human monocytes into effective presenters of an HER2/neu-encoded CTL peptide epitope. *Scand. J. Immunol.*, *45*: 678–682, 1996.
31. Ferlazzo, G., Wesa, A., Wei, W. Z., and Galy, A. Dendritic cells generated either from CD34<sup>+</sup> progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8<sup>+</sup> T cells. *J. Immunol.*, *163*: 3597–3604, 1999.
32. Rongcun, Y., Salazar-Onfray, F., Charo, J., Malmberg, K. J., Evrin, K., Maes, H., Kono, K., Hising, C., Petersson, M., Larsson, O., Lan, L., Appella, E., Sette, A., Celis, E., and Kiessling, R. Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. *J. Immunol.*, *163*: 1037–1044, 1999.
33. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc. Natl. Acad. Sci. USA*, *92*: 432–436, 1995.
34. Brossart, P., Stuhler, G., Flad, T., Stevanovic, S., Rammensee, H. G., Kanz, L., and Brugger, W. HER-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by *in vitro* induced specific cytotoxic T lymphocytes. *Cancer Res.*, *58*: 732–736, 1998.
35. Ostankovitch, M., Le Gal, F. A., Connan, F., Chassin, D., Choppin, J., and Guillet, J. G. Generation of Melan-A/MART-1-specific CD8<sup>+</sup> cytotoxic T lymphocytes from human naïve precursors: helper effect requirement for efficient primary cytotoxic T lymphocyte induction *in vivo*. *Int. J. Cancer*, *72*: 987–994, 1997.
36. Lee, T. V., Anderson, B. W., Peoples, G. E., Castilleja, A., Murray, J. L., Gershenson, D. M., and Ioannides, C. G. Identification of activated tumor antigen-reactive CD8<sup>+</sup> cells in healthy individuals. *Oncol. Rep.*, *7*: 455–466, 2000.
37. Maeurer, M. J., Chan, H. W., Karback, J., Salter, R. D., Knuth, A., Lotze, M. T., and Storkus, W. J. Amino acid substitutions at position 97 in HLA-A2 segregate cytolysis from cytokine release in MART-1/Melan-A peptide AAGIGILTV-specific cytotoxic T lymphocytes. *Eur. J. Immunol.*, *26*: 2613–2623, 1996.
38. Goldrath, A. W., and Bevan, M. J. Selecting and maintaining a diverse T-cell repertoire. *Nature (Lond.)*, *402*: 255–262, 1999.
39. Lalvani, A. R., Brookes, S., Hamblon, J. J., Britton, A. V. S., Hill, A., and McMichael, A. J. Rapid effector function in CD8<sup>+</sup> memory T cells. *J. Exp. Med.*, *186*: 859–865, 1997.
40. Clay, T. M., Custer, M. C., McKee, M. D., Parkhurst, M., Robbins, P. R., Kerstann, K., Wunderlich, J., Rosenberg, S. A., and Nishimura, M. I. Changes in the fine specificity of gp100(209–217)-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. *J. Immunol.*, *162*: 1749–1755, 1999.
41. Lee, K-H., Panelli, M. C., Kim, C. J., Riker, A. I., Betinoti, M. P., Roden, M. M., Fetsch, P., Abati, A., Rosenberg, S. A., and Marincola, F. M. Functional dissociation between local and systemic immune response during anti-melanoma peptide vaccination. *J. Immunol.*, *161*: 4183–4194, 1998.
42. Germain, R. N., and Stefanova, I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol.*, *17*: 467–522, 1999.

# Clinical Cancer Research

## Peptide Priming of Cytolytic Activity to HER-2 Epitope 369–377 in Healthy Individuals

Brett W. Anderson, George E. Peoples, James L. Murray, et al.

*Clin Cancer Res* 2000;6:4192-4200.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/6/11/4192>

**Cited articles** This article cites 39 articles, 24 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/6/11/4192.full.html#ref-list-1>

**Citing articles** This article has been cited by 11 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/6/11/4192.full.html#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, contact the AACR Publications Department at [permissions@aacr.org](mailto:permissions@aacr.org).