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MODIFICATION OF THE *HER2/NEU*-DERIVED TUMOR ANTIGEN GP2 IMPROVES INDUCTION OF GP2-REACTIVE CYTOTOXIC T LYMPHOCYTES

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GP2 (IISAVVGIL), the p654–662 *HER2/neu*-derived tumor antigen, induces HLA-A2-restricted cytotoxic T lymphocytes (CTL) reactive to various epithelial cancers. The binding affinity of GP2 for HLA-A2, however, is very low. To improve the immunogenicity of GP2, we tested 10 different amino acid substitutions into GP2 at the C- and N- terminus. Five out of 10 modified peptides, especially those containing phenylalanine at position 1 (1F), showed a significantly improved binding affinity to HLA-A2. 1F-based modified peptides were well recognized by GP2-specific CTL. These peptides were used to stimulate peripheral blood lymphocytes from HLA-A2 healthy donors using peptide-pulsed autologous dendritic cells (DC). After 3 or more weekly stimulations, CTL activity against GP2 pulsed T2 (T2-GP2) and *HER2/neu*-overexpressing tumor cells was measured in ^{51}Cr release and IFN-γ secretion assays. The modified peptides significantly enhanced GP2-specific CTL activity in some donors. In particular, the peptide with phenylalanine at position 1, leucine at position 2 and valine at position 10 (1F2L10V) maximized the CTL activity against both T2-GP2 and *HER2/neu*-positive tumor cells. Peptide 1F2L10V increased not only the binding affinity to HLA-A2 but also improved recognition of GP2. These data suggest that DC + modified GP2 may improve immune therapies for the treatment of *HER2/neu* overexpressing tumors.

Key words: *GP2; modification; CTL; HLA-A*0201; peptide recognition; binding affinity*

It is well known that CD8+ CTL play an important immunological role in cancer patients.[1] These CTL can recognize short peptides (9–11 amino acid), bound by class I MHC molecules on the surface of tumor cells.[2] Several immunogenic peptides have been identified from cancer cells, especially from melanoma using tumor specific CTL lines or clones. It has been relatively difficult, however, to identify these antigens from epithelial cancer cells, mainly because of the difficulty in establishing tumor specific CTL lines.[3]

HER2/neu is a 185 kd transmembrane protein that is overexpressed in 30–40% of breast and ovarian cancers.[4,5] We identified the HLA-A2-restricted *HER2/neu*-derived peptide GP2 (IISAVVGIL, p654–662) using tumor-associated lymphocytes isolated from patients with ovarian and breast cancers and also showed that GP2 is shared between several distinct types of epithelial tumors including non-small cell lung and pancreatic cancer cells.[6–9] These findings suggest that GP2 may be an attractive candidate for broadly applicable vaccine therapy.

Most of the naturally processed peptides that bind to HLA-A*0201 contain 2 dominant amino acid anchor residues within their sequence: leucine (L) or methionine (M) at position 2 and valine (V) at position 9.[10,11] Many identified tumor-associated antigens including GP2, however, lack at least 1 of these major anchor residues.[12,13] This means that these tumor antigens bind poorly to HLA-A*0201 and only a limited number of antigen-MHC complexes are expressed on the cell surface. A recent study showed that a low density of HLA-A*0201 complexes is closely related to escape from peripheral tolerance.[14]

To improve immunogenicity of relatively low binding peptides, artificial substitutions of amino acids are routinely introduced in non-TCR recognition residues, i.e., positions 1–3 and 9 or 10. Wild-type GP2 lacks both major anchor residues and has a poor binding affinity to HLA-A*0201. Recently, several amino acid modifications were introduced in GP2 and the binding affinity of these modified peptides were tested.[13] Interestingly, none of the modified peptides significantly improved the binding affinity.

To investigate GP2 further, we introduced single, double and triple amino acid substitutions in GP2 and analyzed the effect on the binding affinity to HLA-A*0201. In addition, each modified peptide was tested for induction of CTL using peptide-pulsed autologous dendritic cells.

MATERIAL AND METHODS

Peptide synthesis

GP2-based peptides containing single, double or triple amino acid substitutions were designed according to the HLA-A*0201 binding motif (Table I).[10,11] Peptides were purchased from Genemed Synthesis Inc., (San Francisco, CA). Synthesis was carried out by a standard solid-phase method based on fluorenylmethoxycarbonyl chemistry. Recovered lyophilized peptide was purified by HPLC on C-18 columns and peptide identity and purity (>95%) was demonstrated by mass spectrometry.

Cultured cell lines

The HLA-A2+, peptide transporter associated protein (TAP) deficient T-B cell hybrid T2 cell line and the *HER2/neu* overexpressing ovarian cancer cell line, SKOV3, were purchased from the American Type Culture Collection (Manassas, VA). HLA-A2 transfected SKOV3, SKOV3-A2, was generated by electroporation method. All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin (all from Mediatech, Herndon, VA).

HLA-A2 stabilization assay

T2 cells were pulsed with saturating amounts of individual peptides (100 μg/ml) for 12 hr and then stained with the HLA-A2 specific monoclonal antibody (MAb), BB7.2, by indirect staining. After flow cytometry analysis (FACScaliber, Becton Dickinson, Mountain View, CA) the relative mean channel fluorescence (MCF-R) was calculated as follows; MCF-R = mean channel fluorescence after peptide pulsing (MCFp)/ MCF without peptide pulsing (MCFo). Flu-M1 peptide (GILGFVFTL) was used as a control.

Assessment of the dissociation rate of peptide from HLA-A2

T2 pulsed with saturating amounts of peptide were incubated in RPMI medium with Brefeldin A (BFA) (Sigma, St. Louis, MO) at 10 μg/ml for 1 hr at 37°C and subsequently maintained in medium containing BFA at 0.5 μg/ml. At the indicated times, aliquots were stained with BB7.2 MAb and analyzed by FACS. The % of

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TABLE I – AMINO ACIDS USED FOR MODIFICATION OF GP2 EPITOPES

	Position from N terminus				
	1	2	Residues primarily involved in TCR recognition		9/10
			3	4–8	
Dominant primary anchor residues		L M			V
Preferred or acceptable residues	F	I			L I

Based on sequencing pools of peptides eluted from HLA-A*0201 on cells.

TABLE II – BINDING OF GP2 PEPTIDE ANALOGUS TO HLA-A2

GP2 peptide modification	Sequence	T2 cell surface stabilization assay (MCF-R)	Computer predicted $T_{1/2}$[1]
Parent	IISAVVGIL	1.2	1>
2M	IMSAVVGIL	1.8	3.2
2L	ILSAVVGIL	1.8	4.4
9V	IISAVVGIV	1.3	2.0
2L9V	ILSAVVGIV	2.9	14.5
2M9V	IMSAVVGIV	1.7	10.5
1F9V	FISAVVGIV	3.6	5.4
1F2L	FLSAVVGIL	2.6	12.0
1F2L9V	FLSAVVGIV	2.9	39.2
1F2L10V	FLSAVVGILV	2.2	319.9
Flu-M1[2]	GILGFVFTL	4.0	550.9

Experimental conditions of T2 cell stabilization assay are described in Material and Methods. Underlining indicates MCF–R > 2, which means high stabilizing ability.–[1]Computer analysis was performed by the software "HLA Peptide Binding Predictions," located in the Bioinformatics & Molecular Analysis section on the NIH web site.–[2]Flu-M1 indicated an immunogenic peptite derived from influenza virus matrix protein.

remaining complex was calculated as MCFp minus MCFo and normalized to the maximum level (at time zero) of MCF for each peptide.

HLA typing and subtyping

HLA-A2 status of healthy donors and patients was evaluated by staining of lymphocytes with BB7.2 and the HLA-A2-specific MAb MA2.1. The HLA-A2 subtype was determined by PCR, as previously described.[15]

Generation of a GP2 specific CTL line

Mononuclear cells (MNCs) were separated from malignant chest fluid of a HLA-A*0201 breast cancer patient by density centrifugation on 100% Ficoll (Histopaque, Sigma). After simulation with solid-phase anti-CD3 MAb for 24 hr, MNCs were cultured in RPMI with 10% human AB serum supplemented with 50 IU/ml of IL-2 (Kindly provided by Amgen, Thousand Oaks, CA) and 10 ng/ml of IL-7 (Endogen, Rockford, IL). MNCs received 3 weekly stimulations with GP2 pulsed onto irradiated (10,000 rads) T2 cells at a 10:1 responder to stimulator ratio and were then tested for specific recognition of GP2.

In vitro *generation of DC*

DC were generated from peripheral blood (PB)MNCs from normal donors ($n = 4$) by centrifugation on Ficoll. PBMNCs were plated in 10 cm^2 culture dishes (1×10^8 cells/dish) and monocyte-enriched adherent cells were observed after a 4 hr incubation at 37°C. The non adherent cells were removed and cryopreserved and the adherent cells were cultured in the presence of 800 U/ml recombinant (r)-granulocyte-macrophage colony-stimulating factor (GM-CSF) and 400 U/ml rIL-4 (both from Endogen) in complete medium supplemented with 10% human AB serum. On Day 6, 100ng/ml LPS (Sigma) was added in medium and on Day 7 the cytokine-treated cells were harvested and used as mature DC.

In vitro *CTL induction using modified GP2 peptides and DC*

After treating with a mild acidic buffer (0.13 M citric acid, pH 3.0) for 2 min, the DC were pulsed with 100 μg/ml of each peptide for 1 hr on ice. The peptide-loaded DC were irradiated with 5,000 rads and mixed with MNCs at a ratio of 1:10 in the presence of 10 ng/ml rIL-7 in complete medium supplemented with 10% human AB serum. On Day 7, the responder cells were restimulated with peptide-loaded DC in medium with 10 ng/ml rIL-7 and 50 U/ml rIL-2. Responder cells received at least 3 weekly stimulations.

Evaluation of antigen recognition by a CTL line and peptide-induced CTL

Standard ^{51}Cr release assays and cytokine (IFN-γ) release assays were performed to evaluate the recognition of peptide and *HER2/neu*-expressing tumor cells. ^{51}Cr-labeled T2 cells were pre-incubated with 100 μg peptide for 1 hr at 37°C. These cells and ^{51}Cr-labeled T2, SKOV3 and SKOV3-A2 cells were then used as targets in cytotoxicity assays (2,000 targets/well). Target cells and effector cells were coincubated for 4 hr at 37°C and the radioactivity in supernatants was determined with a gamma counter. The percent specific lysis of target cells by CTL was calculated. Spontaneous release never exceeded 25% of the maximum. In cytokine release assays, 10^5 CTL were co-incubated with 10^5 tumor cells or peptide-loaded T2 cells for 24 hr at 37°C. The concentration of human IFN-γ in co-culture supernatants was then determined using a commercially available ELISA kit (Biosource, Camarillo, CA).

RESULTS

*Amino acid substitutions of GP2 can increase their binding affinity to HLA-A*0201*

Each of the 10 GP2 analogues was assessed for its binding affinity to HLA-A2 by the HLA-A2 stabilization assay using T2. The data were compared to the predicted half-life values following a computer algorithm (Table II).[16] Both the HLA-A2 stabilization assay and the computer algorithm showed that parent GP2 is a weak binder. Single amino acid substitutions (2L, 2M and 9V) resulted in moderate improvement in binding affinity. Double and triple amino acid modifications, especially 2L9V and 1F-based modifications (1F9V, 1F2L, 1F2L9V, 1F2L10V) markedly enhanced the peptide binding affinity to HLA-A2 and the stability increased more than 2 times as compared to unmodified GP2.

Recognition of modified GP2 by a GP2-specific CTL line

To assess the relative antigenic activity of each modification and cross-recognition, the recognition of T2 + modified peptide by a GP2-specific CTL line was evaluated by IFN-γ secretion (Fig. 1). Almost all modified peptides except for 9V enhanced antigenicity compared to GP2. In particular, 2L and 1F based modifications were strongly recognized by the GP2-specific CTL line. This cell line also recognized the *HER2/neu*-overexpressing SKOV3-A2, but not the HLA-A2-negative SKOV3.

*Assessment of the dissociation rate of selected modified GP2 peptides from HLA-A*0201*

Based on the binding and recognition studies, we focused on 4 1F-based modifications that had both a relatively high binding affinity to HLA-A*0201 (MCF-R > 2)[17] and were strongly recognized by a GP2 specific CTL line. Despite the good recognition of the 2L peptides (Fig. 2), this peptide was not further studied because of its inability to significantly improve the T1/2 of peptide-HLA-A2 complexes and induction of CTL over wild-type GP2.[9,13] HLA-A*0201 complexes formed with GP2 were extremely unstable, reaching background levels within 1 hr of incubation at 37°C (Fig. 2). Despite the comparatively high binding

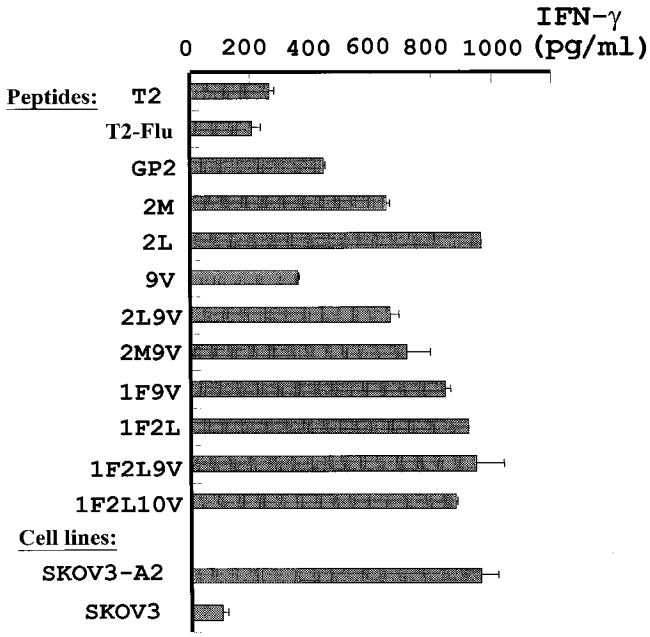

FIGURE 1 – Recognition of GP2 peptide analogues by a GP2-specific CTL line derived from breast cancer associated lymphocytes. Recognition of T2 + Peptides was evaluated by IFN-γ secretion (ELISA). HLA-A2 restriction of this CTL line was assessed by testing the CTL line against SKOV3 and SKOV3-A2. Results ± SEM are shown.

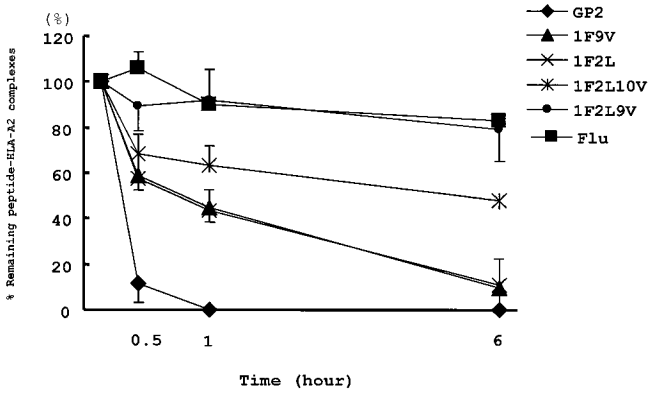

FIGURE 2 – Assessment of dissociation rate of GP2 analogues from HLA-A2. Five modifications were selected based on the results of the T2 stabilization assay. Experimental conditions of assessment of dissociation rate are described in Material and Methods. Results of 3 experiments ± SEM are shown.

affinity, complexes formed with 1F9V and 1F2L only slightly improved the HLA-A2/peptide stability. 1F2L10V formed complexes that were significantly more stable with a T1/2 of around 7 hours. 1F2L9V even more strongly stabilized the peptide-HLA-A2 complexes. This stability was equal to complexes formed with the Flu-M1 peptide (positive control). The calculated half-life time of each modification are shown in Table III.

Screening of modified GP2 peptides for CTL induction

The ability of the modified peptides to induce GP2-specific CTL was evaluated by stimulating PBMC derived from 3 consecutive HLA-A*0201 healthy donors with autologous irradiated DC pulsed with modified peptide. After 4 or 5 times weekly stimulations, recognition of GP2 peptide by CTL was measured by ^{51}Cr release (HD1 and HD2) or IFN-γ ELISA (HD3). The CTL induction study demonstrated that CTL reactive against the parent GP2 epitope were induced most efficiently in all 3 donors with 1F2L10V modified peptide (Table IV). The other 1F-based modifications did not consistently improve induction of GP2-specific CTL compared to parent GP2.

TABLE III – HALF LIFE OF GP2 PEPTIDE ANALOGUE-HLA-A*0201 COMPLEXES[1]

GP2 peptide modification	$T_{1/2}$ (hr)
Parent	0.16
1F9V	1.93
1F2L	2.12
1F2L10V	7.58
1F2L9V	20.84
Flu-M1	21.58

[1]The half life time of the peptides-HLA-A*0201 complexes was determined by the dissociation rate of the peptides from HLA-A*0201 molecules.

TABLE IV – RECOGNITION OF GP2 BY CTL INDUCED WITH MODIFIED PEPTIDES

GP2 peptide modification	HD1 %specific lysis (20:1 E:T) (4 wks)	HD2 %specific lysis (20:1 E:T) (5 wks)	HD3 IFN-γ (pg/ml) (4 wks)[1]
Parent	25[2]	4	52
1F9V	6	3	57
1F2L	19	1	99
1F2L9V	10	18	75
1F2L10V	**34**	**49**	**162**

Peptide-specific CTL were generated by weekly stimulation of T cells with autologous DC pulsed with peptide. Recognition of parent GP2 peptide was evaluated using GP2-loaded T2 cells; in cytotoxicity assays (HD1 and HD2), or IFN-γ secretion assays (HD3). The percent lysis or IFN-γ secretion in response to T2 only has been subtracted. –[1]CD4+ T cell depletion was performed before entering stimulations.–[2]Values in parentheses indicate background % cytolysis (HD1 and HD2) or IFN-γ release (HD3) when T2 cells without exogenous peptide were used.

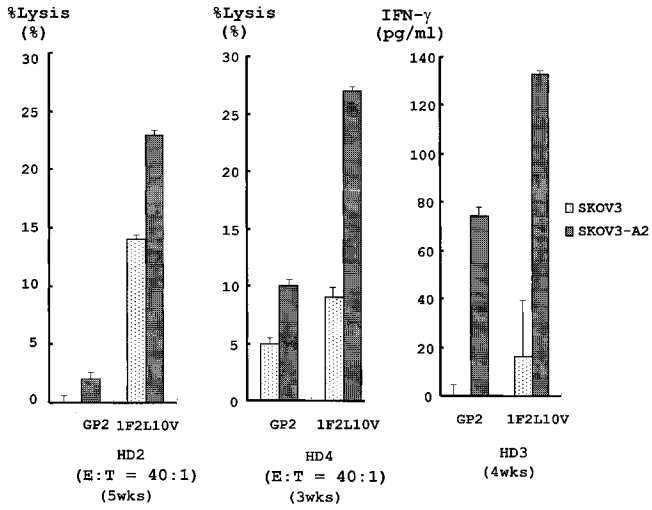

FIGURE 3 – Recognition of ovarian cancer cells by CTL induced with GP2 parent and 1F2L10V. CTL were induced by weekly stimulation with autologous DC pulsed with GP2 or its modification,1F2L10V. Recognition of carcinoma cells was evaluated using the *HER2/neu* overexpressing ovarian cancer cell line, SKOV3 and a HLA-A2 transfectant, SKOV3-A2, by standard ^{51}Cr release assay for HD2 and HD4 and IFN-γ secretion for HD3. Results ± SEM are shown.

Induction of GP2-specific, tumor-reactive CTL by in vitro *stimulation with 1F2L10V*

To further examine the CTL induced by in vitro stimulation with 1F2L10V modified GP2, CTL were tested against SKOV3, the *HER2/neu* overexpressing ovarian cancer cell line and SKOV3-A2, a HLA-A*0201 transfectant. Compared to GP2, the 1F2L10V peptide induced strong CTL activity against SKOV3-A2 in all 3 healthy donors (Fig. 3). The recognition of SKOV3-A2 appeared HLA-A2 restricted because the HLA-A2-negative SKOV3 induced significantly lower levels of recognition. Together the data suggest that the 1F2L10V modification of GP2 is the best peptide among the modified peptides tested in our study with regard to induction of GP2-specific and tumor-reactive CTL.

DISCUSSION

A large variety of tumor antigens encoding T cell epitopes have been identified.[18] Most of these antigens are derived from self proteins. The antigenic peptides, which are potential targets for immune therapy, usually contain subdominant amino acids in at least one of the anchor positions and have an intermediate to low binding affinity for the MHC class I molecule.[12,19] In the case of self-antigens, one would expect that peptides with a high binding affinity for MHC induce tolerance due to thymic selection. In contrast, poor binders may permit T cells to escape from negative selection and these peptides and T cells may play important roles in tumor-specific immunity.[14,20] The poor peptide-MHC binding makes it difficult to induce peptide-specific T cell immunity, however, especially from PBL.[21] To overcome this problem, amino acid substitutions have been tested in several tumor antigens, such as *NY-ESO-1*, *gp100* as well as *MART-1*.[22,23] Several modifications successfully improved the binding affinity for HLA and immunogenicity.

Two reports and our current results demonstrate that GP2 has a low binding affinity for the HLA-A*0201 molecule.[13,24] Initially, we considered the lack of dominant amino acids at both anchor sites the reason for poor binding. This means that double amino acid substitutions at positions 2 and 9 would give it a more ideal binding motif. According to a recent investigation, however, single or double amino acid substitutions at anchor residues of GP2 did not significantly increase its binding affinity; instead, the crystallographic structure indicated that the center of the GP2 peptide is "disordered" and does not make stabilizing contacts with the peptide-binding cleft.[13] In our experiments, single or double amino acid modifications at the anchor positions did not significantly improve stabilization of the HLA-peptide complex (data not shown). To further examine modifications of this unique peptide, we introduced a third amino acid substitution at position 1. Surprisingly, 1F-based amino acid modifications improved both the binding affinity to HLA-A2 and recognition by GP2-specific CTL even when 1 of the 2 anchor residues still included a subdominant amino acid. According to the algorithm, the triple amino acid substitution 1F2L10V should have the highest binding by far (Table II), but the dissociation assays showed that although this modification significantly stabilized the complex, it was similar to that of 1F2L9V modification.[16]

The most important assay to select a suitable modification of GP2 is to screen each modification for the ability to induce GP2-specific CTL. According to our binding and recognition assays, 1F2L9V or 1F2L10V are the best immunogenic modifications of GP. Although 1F2L9V was the better binder to HLA-A2, 1F2L10V was the better CTL inducer. It is difficult to explain why 1F2L10V is superior to 1F2L9V in terms of CTL induction. A recent study demonstrated that a short term cultured CTL line consisted of a mix of heterogeneous CTL clones, with each clone having different biologic features and TCR repertoire.[25] We speculate that each GP2 modification may stimulate and expand a slightly different subset of CTL clones with overlapping functional characteristics. Consequently, the various CTL lines may differentially recognize the wild-type GP2. This means a modification that increases both binding affinity and recognition by GP2-specific CTL does not necessarily improve induction of GP2-specific CTL. Similar findings were observed with modifications of the melanoma antigen gp100.[19]

Recently, HLA-A2-H2K^b (A2K^b) transgenic mouse were utilized to analyze the immunogenicity of modified GP2. Serody et al.[24] demonstrated that DC pulsed with the 2L modified GP2 injected into A2K^b transgenic mouse did not enhance a CTL response compared to DC pulsed with GP2. Although the A2K^b transgenic mouse may be a useful system to analyze HLA-A2 binding peptides, it should be noted that the human *erb/B2* gene encodes a completely different amino acid sequences compared to the murine *erb/B2* gene in the GP2 region.[26] This indicates that GP2 does not induce tolerance in the mouse immune system and the immunological reaction against GP2 could be different between human and mouse.

HER2/neu gene is an attractive target molecule to utilize in tumor immune therapy because of its overexpression in many types of epithelial tumors. So far, several immunogenic peptides have been identified from *HER2/neu*.[27,28] Most of them have an intermediate to strong binding affinity to HLA-A*0201. In particular, E75 (p369–377), a so called immunodominant peptide, has been well characterized.[27] Interestingly, in 1 human trial using the E75 peptide, a peptide-specific CTL reaction could be easily generated. These CTL, however, failed to recognize breast and ovarian tumor cell lines.[29] In another trial, both E75 and GP2 were tested but only CTL responses against E75 could be detected. These CTL also recognized a *HER2/neu* overexpressing HLA-A2+ ovarian tumor cell line.[30] Compared to E75, which has a strong binding affinity to HLA-A2, GP2 has a relatively poor binding affinity that might inhibit effective CTL induction *in vivo*. The poor binding of GP2, however, may suggest that GP2-specific precursor CTL exist and have not been deleted. In fact, there are relatively large numbers of GP2-specific precursor CTL in advanced colon cancer patients.[31] Our earlier studies showed that GP2-CTL could be generated from ovarian and breast cancer patients.[6,8] In our current study, a strong GP2-specific CTL reaction was elicited by modified GP2-pulsed DC from PBL from healthy individuals. Thus, GP2-specific precursor CTL may be present in normal donors and cancer patients at varying frequencies and modification of GP2 could efficiently induce GP2 specific CTL from those precursors.

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