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In vitro generated cytolytic T lymphocytes reactive against head and neck cancer recognize multiple epitopes presented by HLA-A2, including peptides derived from the p53 and MDM-2 proteins

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Abstract

In previous studies, we were successful in generating HLA-A2-restricted CD8+ CTLs reactive with head and neck carcinomas (HNCs) in 4/10 cases using traditional mixed lymphocyte tumor cultures (MLTCs) employing a semi-allogeneic HLA-A2+ HNC cell line, PCI-13, as the stimulator of normal HLA-A2+ donor T lymphocytes. However, these T cell lines contained only 1-1.5% HLA-A2-restricted, tumor-reactive CD8+ CTLs, as assessed by both limiting dilution and IFN-gamma ELISPOT assays. In order to increase the success rate in generating such HNC-reactive CTL lines, we modified the procedure to allow for T cell crosspriming by autologous DCs pulsed with PCI-13 lysates. In all three attempts, HLA-A2-restricted effector T cell lines were obtained that contained PCI-13-reactive CD8+ T cells at frequencies as high as 1 in 6. These cultured bulk lines recognized at least five predominant HLA-A2-restricted epitopes based on ELISPOT fingerprinting of HPLC-fractionated, naturally presented PCI-13-derived peptides. Two of these epitopes appear to be derived from the p53 and MDM-2 proteins overexpressed by the PCI-13 cell line. Interestingly, the synthetic wild type sequence $p53_{264-272}$ and MDM-2_{53-61} peptides were able to drive *in vitro* generation of tumor-specific CTLs from the PBMCs of normal HLA-A2+ donors. However, this MDM-2 peptide was not able to elicit responses from HLA-A2+ patients with HNC in short-term *in vitro* cultures. Overall, these data suggest that tumor lysate-loaded DCs elicit a broad repertoire of CTL responses, some of which are directed against peptides derived from cell cycle regulatory proteins that may prove to be of clinical significance in the therapy of HNC.

Introduction

Human cancers of the head and neck are often infiltrated by mononuclear cells (1, 2, 3, 4, 5). However, these tumor-infiltrating lymphocytes (TILs) are largely dysfunctional and many have been shown to undergo apoptosis *in situ* (3, 5). In some instances, however, clonotypic T cell expansions within HNC lesions have been reported to

be associated with a better clinical prognosis and tumor regression (6, 7, 8). Absence of signaling defects in TILs, as evidenced by normal zeta chain expression, was found to predict a significantly better 5-year survival in patients with oral carcinoma (5). Furthermore, adoptive therapy with tumor-reactive T cells has been demonstrated to eradicate HNC *in situ* (9). These data strongly suggest that the development of vaccines designed to augment the number, tumor localization and maintenance of HNC-specific T cells may be important and lead to promising therapeutic strategies for patients with HNC (1, 10). Vaccines capable of eliciting strong, tumor-specific cytolytic T cell responses in patients with head and neck cancer predicated upon the use of semi-allogeneic tumor cells might prove both efficacious and logistically desirable.

Tumor-reactive cytotoxic T lymphocytes may be activated *in situ* via two principal antigen presenting cells: tumor cells themselves or host dendritic cells that cross-present antigens acquired via the uptake of tumor debris or apoptotic bodies (11, 12). In general, tumor cells are not considered to be effective APCs, because they either downregulate MHC molecules or antigenic epitopes (13). In addition, their antigen-processing machinery may be defective (14). Nevertheless, there is ample evidence suggesting that tumors can function as APCs and are recognized by tumor-specific immune effector cells. The role of DCs in immune responses targeted to tumors has recently been emphasized, primarily their ability to mediate "epitope spreading" (15). In oral carcinoma, the presence of DCs in the tumor was found to be an independent and highly significant biomarker of 5-year survival (1).

In the current study, we have extended our evaluation of the functional capacity of each of these two APC types as components of *in vitro* vaccines designed to drive the expansion of HNC-reactive CTLs from normal HLA-A2+ donors. An HLA-A2+ HNC cell line, PCI-13, could serve as both the direct presenter of peptides to CTLs and as a source of protein lysate that was fed to autologous DCs to allow for crosspresentation. Both direct- and cross-presentation methods were effective in promoting the *in vitro* expansion of effector HNC-reactive CTLs, although the response rates were higher when DCs were employed. Subsequent dissection of the nature of HNC epitopes recognized by these CTLs suggests that the *in vitro* vaccines elicit a polyspecific cellular immune response and that peptides from the known tumor-associated antigen p53 and from the MDM-2 protein may serve as targets for CD8+ effector cells. Given the limited panel of defined T cell epitopes in HNC (16, 17, 18), the application of either tumor lysates or synthetic peptides pulsed on autologous DCs could be considered for the therapy of patients with HNC.

Results

CTL generation using autologous DCs pulsed with PCI-13-derived antigens

While we had previously been able to generate CTLs against HNC in mixed lymphocyte-tumor cultures (MLTCs) in 4 of 10 attempts (data not shown), we sought a method to further improve the efficiency of expanding specific CTLs *in vitro* and to reduce concerns associated with the allogenic nature of the PCI-13 stimulator cell line used in our MLTCs. We prepared DCs autologous to the responder HLA-A2+ PBMCs and pulsed them with antigens derived from PCI-13 lysates, prior to use as *in vitro* stimulators. We had previously demonstrated that the use of this antigen lysate format for loading DCs did not result in the discernable activation of alloreactive CTL clones from autologous T cell responders *in vitro* (19). In all three attempts at CTL generation, we were able to establish, expand and test CTL lines (designated CTL-DC#1 to -DC#3), when using lysate-pulsed DCs in the first two *in vitro* sensitization cycles (Figure 1). The levels of cytolysis directed against the PCI-13 target cell line were consistently high (i.e. >50% lysis at effector:target cell ratios exceeding 8:1), and recognition was blocked by inclusion of the anti-HLA-A2 mAb MA2.1. The CTL lines were also tested using the IFN-gamma ELISPOT to

assess the frequency of specific CTL responders against the HLA-A2+ PCI-13 cell line and other HLA-A2+ HNC cell lines (Table 1). In general, the greatest frequencies of specific CD8+ T cells were observed against the PCI-13 target cell line, but all three lines also contained lower frequencies of T cells reactive against other HLA-A2+ HNC cells, supporting tumor cell presentation of shared tumor antigens/epitopes. Responses to the PCI-13 target cell line and to the other CTL-activating HLA-A2+ tumor cell lines were partially blocked by mAbs W6/32 and MA2.1 (Figure 1 and Table 1). These T cell lines did not recognize the HLA-A2-negative HNC target cell line SCC74 or the HLA-A2+ HR gastric carcinoma cell line.

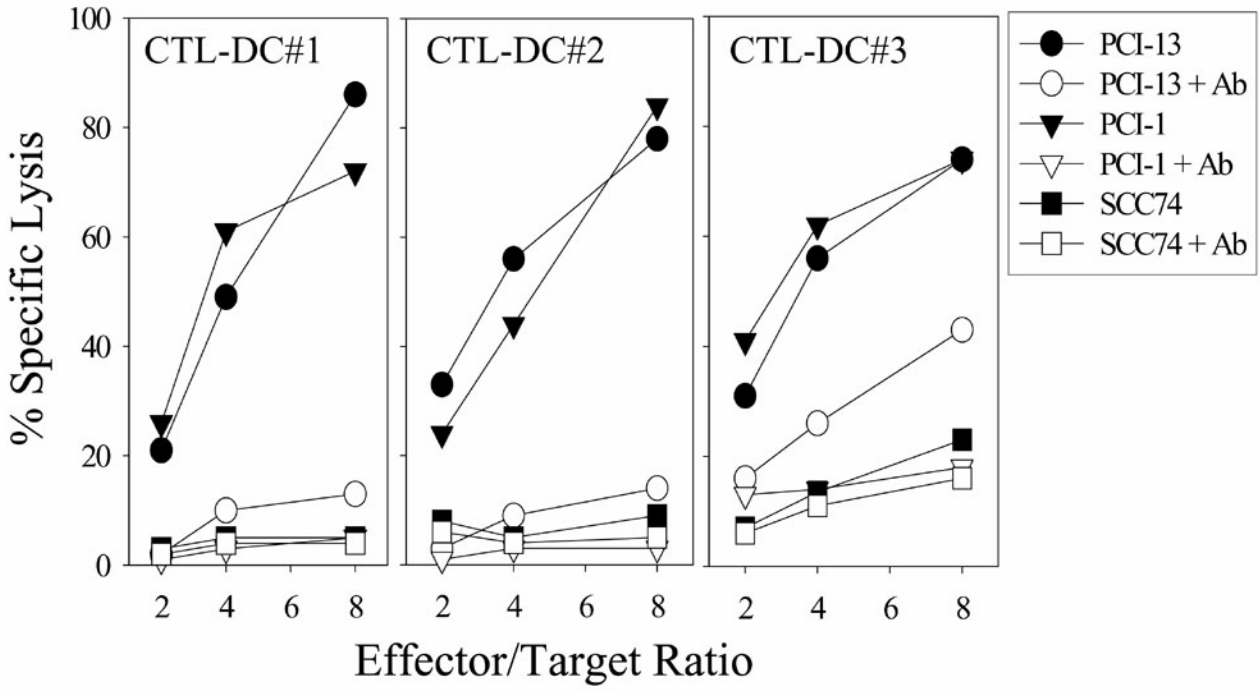

Figure 1. CTL lines generated from HLA-A2+ normal donors after *in vitro* stimulation with autologous DCs pulsed with PCI-13 lysates mediate the HLA-A2-restricted killing of HNC targets. Normal HLA-A2+ donor PBMCs were stimulated twice with autologous DCs preloaded with lysate derived from the PCI-13 cell line and subsequently with irradiated PCI-13 cells on a weekly regimen as outlined in the Materials and Methods. On day 32-37 of culture, CD8+ T cells were enriched and analyzed for their ability to lyse PCI-13 target cells in the absence or presence of blocking anti-HLA-A2 mAb MA2.1 in 4 h cytotoxicity assays at the indicated E/T ratios. Targets included the HLA-A2+ HNC cell lines PCI-13 and PCI-1, and the HLA-A2-negative HNC cell line SCC-74. Data are from one representative assay of 2 performed.

The CTL lines recognize multiple naturally processed and HLA-A2-presented peptides derived from proteins expressed by HNC cell lines

We next decided to "fingerprint" the range of peptide epitopes recognized by the HLA-A2 restricted CTL-DC#1 line by extracting naturally processed and cell surface-expressed epitopes from the HLA-A2+ HNC cell lines PCI-13 and PCI-1, and the HLA-A2-negative HNC cell line SCC-74 (see Table 2). Total MHC class I-bound peptides were eluted from viable PCI-13 cells grown in cell factories by brief treatment with iso-osmotic pH 3.0 citrate-phosphate buffer using methods previously developed in our laboratory (20, 21, 22). Isolated peptides were resolved on reverse-phase high performance liquid chromatography (RP-HPLC), lyophilized to remove organic solvents and reconstituted in PBS. Finally, peptides from individual HPLC fractions were analyzed for their ability to stimulate IFN-gamma production from CTL-DC#1 when presented by the HLA-A2+ T2 cell line in ELISPOT assays (Figure 2). At least four major and one minor species of peptide epitopes derived from the PCI-13 cell line were identified (peak fractions 26, 30, 38, 48 and 54) that were able to activate the CTL-DC#1 line, with

approximately 6% of all CTLs recognizing the peptide(s) species present in HPLC fraction 48. These same bioactive peak fractions were also identified when the HLA-A2+ PCI-1, but not the HLA-A2-negative SCC-74 cell line (data not shown), was used as the source for peptide extraction (Figure 2). Indeed, no SCC74-derived HPLC fractions promoted more than 4 spots/well (i.e. 4 spots/2000 CD8+ T cells) from either responder CTL line evaluated. These data strongly support the shared nature of the majority of these HLA-A2-associated peptides recognized by CTL-DC#1, although the stoichiometric abundance of each species varies with respect to the HNC cell line used for elution; i.e. HPLC fraction 38 peptide(s) appear more abundant in eluates from the PCI-1 than from the PCI-13 cell line. CTL-DC#2, generated from an unrelated HLA-A2 donor, also recognized peptides contained in HPLC fractions 26, 48 and 54, but not fractions 30 and 38, derived from PCI-13 and PCI-1, but not SCC-74 (Figure 2 and data not shown). Interestingly, CTL-DC#1 and CTL-DC#2 also differentially recognized novel shared peptides eluting in HPLC fractions 17, 30 and 38 derived from PCI-13 and PCI-1 (but not SCC-74) cell lines.

Table 1. Frequency of IFN-gamma-producing T cells in CTL lines stimulated with different HLA-A2+ target cells in ELISPOT assays.[a]

Effector Cell	Target Cell Line Evaluated					
	PCI-13	PCI-1	PCI-30	OSC-19	HR	None
CTL-DC#1 (W6/32)	17 (33)	1,000 (5,000)	25,000 (>100,000)	1,667 (2,778)	12,000	20,000
CTL-DC#2 (W6/32)	56 (278)	179 (313)	132 (278)	132 (500)	1,200	1,500
CTL-DC#3 (W6/32)	6 (8)	25 (30)	10,000 (20,000)	2,857 (6,667)	NT[b]	3,333

[a]The CTL lines were tested in 20 h ELISPOT assays in response to no target cells or to the HLA-A2+ HNC lines (PCI-13, PCI-1, PCI-30, OSC-19) or gastric carcinoma (HR) target cell lines listed. The ratio of CTL:target cells was 10:1 (i.e. 100,000 CTL: 10,000 target cells) in all assays. The data are reciprocal frequencies (1/X) of IFN-gamma producing cells. To confirm that IFN-gamma production resulted from MHC class I-restricted interaction, W6/32 Abs were added to duplicate assays, and the reciprocal frequency of IFN-gamma producing cells in the presence of this Ab is shown in parentheses.
[b]NT, not tested.

Table 2. HLA typing of tumor cell lines and PBMC obtained from normal blood donors.

Cell line	Locus[a]				
	A	B	C	DR	DQ
Tumor cell lines					
PCI-13	2, 3	7, 35	w12, w15	4(0401), 7(0701)	w2, w3
PCI-1	2, 3	7, 39	4, 7	53	2, 8
PCI-30	2, 28	49, 53	ND	2, 5	w1, w3
OSC-19	2	7	7	1	5
PCI-23	1	8, 44	ND	3, 7	w2
PCI-4B	24, 26	35, 44	w4, w14	1, 3	w3
SCC-74	33	15, 51	4, 16	1, 13	6
HR (Adeno, CA)	1, 2	8, w56	ND	3	ND
MEL526	2, 3	50, 62	ND	ND	ND
T2	2	-	-	-	-
CTL lines established from PBMCs of normal donors					
CTL-DC#1	1, 2	8, 35			
CTL-DC#2	1, 2	44, 55	6		
CTL-DC#3	2, 32	44, 50	5, 6		

[a]ND, not determined.

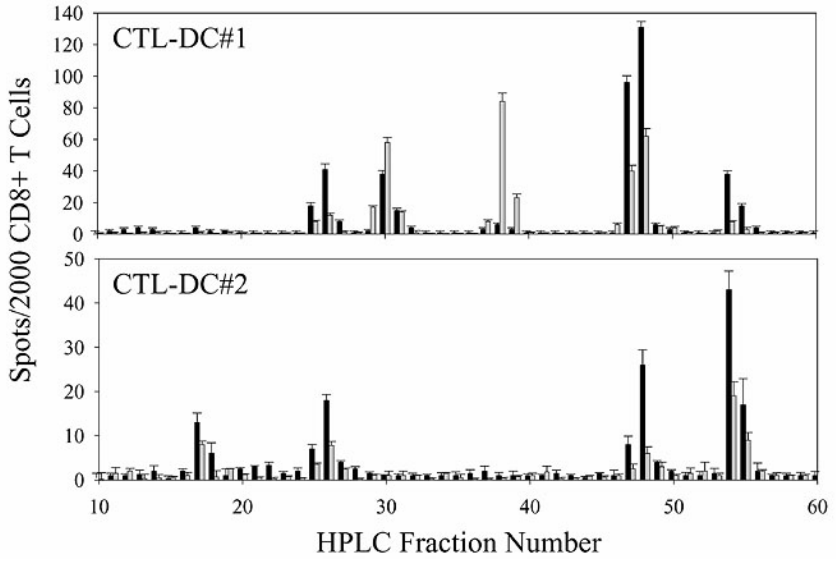

Figure 2. HPLC fingerprinting of natural peptide reactivity of the CTL lines. Total MHC class I-bound peptides were isolated from approximately 10^9 PCI-13 (filled bars) or PCI-1 (open bars) tumor cells and separated individually on RP-HPLC as described in Materials and Methods. T2 cells (expressing only the HLA-A2 class I allele) were pulsed with peptides from individual HPLC fractions and analyzed for their ability to promote IFN-gamma secretion from CD8+ CTLs (CTL-DC#1 or CTL-DC#2) in 20 h ELISPOT assays. The assays were performed in triplicate and spots were counted using a Zeiss autoimager. The data are reported as the mean ± SD for one representative analysis of 3 independent assays performed.

Attempts to sequence PCI-13-derived peptide epitopes using mass spectrometry

Individual HPLC fractions (i.e. 26, 30, 38, 48, 54) containing PCI-13 derived peptides recognized by CTL-DC#1 were submitted to the UPCI Protein Sequencing Facility for mass spectrometric analyses. While a number of stoichiometrically dominant peptides were sequenced and synthesized, these peptides were not recognized by any of our HNC-reactive CTL lines when presented by the (HLA-A2+) T2 cell line *in vitro*. This suggests that the relevant bioactive epitopes represent minor species in the HPLC fractions recognized by our CTL lines.

The PCI-13-reactive CTL-DC#1 line recognizes HLA-A2-presented peptides derived from the p53 and MDM-2 proteins

As an alternative to the mass spectrometric-based search for PCI-13-derived epitopes, we directly analyzed the antigenicity of peptides derived from tumor-associated proteins, which we knew or hypothesized were overexpressed by the PCI-13 cell line. Recent studies by our group have documented the accumulation of mutant p53 tumor suppressor gene products in the PCI-13 cell line expressing a point mutation in codon 286 (16). Since the overexpression of p53 has been reported to result in the causal elevation of p53-dependent cell cycle regulatory and DNA repair proteins such as MDM-2 (23, 24, 25), we anticipated that MDM-2 would be expressed by the PCI-13 cell line at supratypic levels. Western blot and immunohistochemistry analyses of the PCI-13 cell line confirmed overexpression of both p53 and MDM-2 vs. control cells (Figure 3).

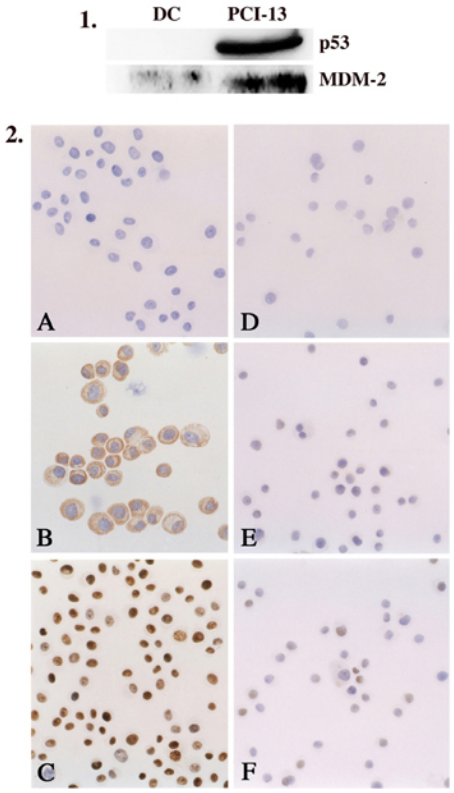

Figure 3. PCI-13 overexpresses both the p53 and MDM-2 proteins. Total PCI-13 (or control DC) lysates were separated on reducing 10% SDS-PAGE gels, Western blotted using specific anti-human p53 and MDM-2 mAbs, and imaged on film by chemiluminescence (panel 1). Immunohistochemistry for p53 and MDM-2 was also evaluated for PCI-13 (and control keratinocyte) cultured cells (panel 2). Cells stained with IgG control (panels 2A and 2D), anti-MDM-2 Abs (panels 2B and 2E) and anti-p53 Abs (panels 2C and 2F) are shown at 200X the original magnification.

Using an algorithm available on the Internet (26, 27), we analyzed, selected and synthesized a series of predicted HLA-A2 binding peptides from the overexpressed MDM-2 protein (Table 3). CD8+ T cells isolated from the CTL-DC#1 line were then analyzed for their capacity to recognize these peptides as well as the previously reported HLA-A2-presented $p53_{264-272}$ peptide (1, 6, 16, 23, 28, 29) in IFN-gamma ELISPOT assays (Figure 4). Approximately 1 in 25 CTLs recognized the MDM-2_{53-61} peptide, while 1 in 100 CTLs recognized the $p53_{264-272}$ and MDM-2_{48-56} peptides when presented by the T2 cell line in ELISPOT assays. This recognition was efficiently blocked by the addition of the anti-HLA-A2 mAb MA2.1 (Figure 4) during the ELISPOT culture period, supporting the HLA-A2 restricted nature of CTL recognition.

Table 3. Synthetic peptides evaluated in this study.[a]

Protein	AA Residues	Sequence	Predicted Binding Score[b]
MDM-2	48-57	YTMKEVLFYL	371
MDM-2	53-61	VLFYLGQYI	361
p53	264-272	LLGRNSFEV	686

[a]The BAX, p21, MDM-2 and GADD45 protein sequences were obtained and analyzed for potential HLA-A2 binding peptides using a computer algorithm (see Materials and Methods). Two high-scoring sequences were synthesized. The defined p53 264-272 naturally processed and HLA-A2-presented epitope was also produced for CTL specificity analysis.
[b]Based on an estimate of the half-life stability of an HLA-A2 complex binding the indicated peptide sequence.

$p53_{264-272}$ and MDM-2_{53-61} appear to represent naturally processed PCI-13 peptide epitopes

Each of these bioactive peptides was analyzed for its elution time under the same RP-HPLC conditions used to isolate the natural PCI-13 peptides in Figure 4. The $p53_{264-272}$ peptide, which eluted in fraction 53, and the MDM-2_{53-61} peptide, which eluted in fraction 48 approximated similar retention times to those associated with the natural PCI-13 peptides (data not shown). To further confirm the potential identity of the natural and synthetic species, cold-target cytotoxicity analyses were performed (Figure 5). Radiolabeled T2 cells preloaded with the natural PCI-13 peptides eluted in HPLC fractions 48 and 54 were cultured with the CTL-DC#1 line, in the absence or presence of unlabeled T2 cells preloaded with the synthetic p53 or MDM-2 peptides. Natural PCI-13 peptide(s) recognized by this CTL line in fraction 48 were effectively cold-target inhibited by the synthetic MDM-2, but not the p53 peptide, while the ability of the CTL-DC#1 line to recognize T2 cells pulsed with fraction 54 peptides was only blocked by the synthetic p53 peptide. CTL recognition of all other PCI-13 bioactive fractions (i.e. 26, 30 and 38) was not inhibited by any synthetic peptide evaluated in this study.

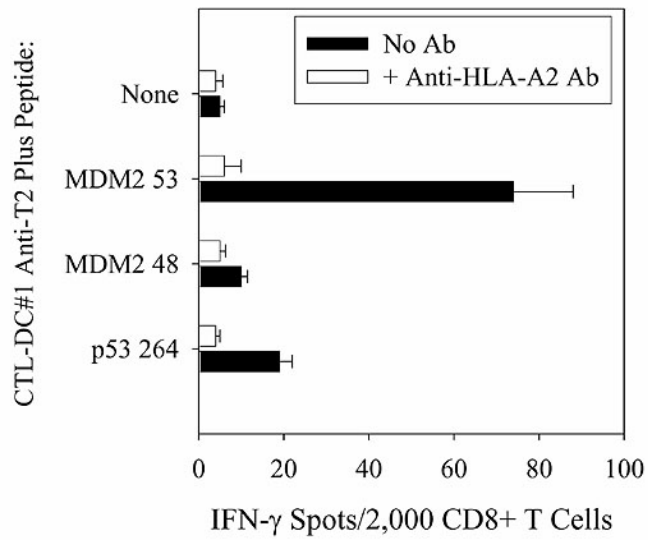

Figure 4. The CTL-DC#1 line recognizes predicted HLA-A2 binding peptides derived from the p53-dependent protein MDM-2. Predicted HLA-A2 binding peptides derived from MDM-2 (see Table 3) and the known $p53_{264-272}$ epitope were evaluated for their ability to activate CD8+ T cells isolated from the CTL-DC#1 line when presented by the HLA-A2+ T2 cell line in IFN-gamma ELISPOT assays. The anti-HLA-A2 mAb MA2.1 was added (5 µg/well) to replicate sets of wells to validate the HLA-A2-restricted nature of peptide recognition by the CTLs. Assays were performed in triplicate and spots were counted using a Zeiss autoimager. The data are reported as the mean ± SD for one representative analysis of 3 independent assays performed.

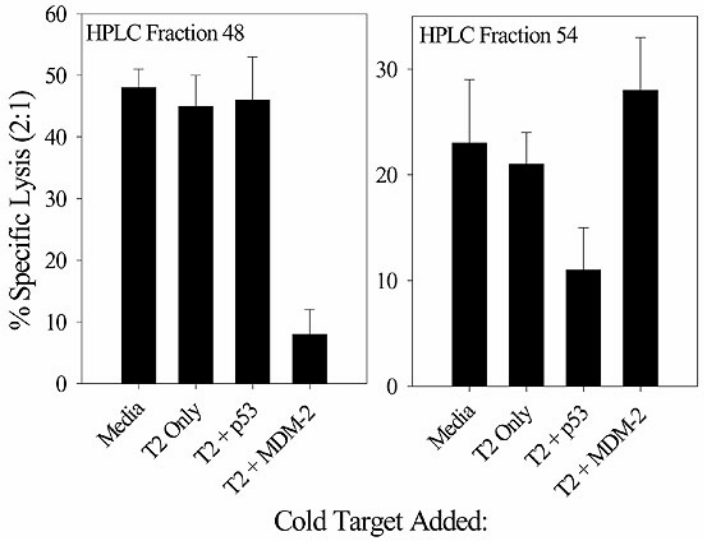

Figure 5. The $MDM\text{-}2_{53-61}$ and $p53_{264-272}$ peptides specifically cold target inhibit CTL recognition of HPLC fractions 48 and 53 respectively. ^{51}Cr-labeled T2 cells were pre-loaded with peptides from either PCI-13 HPLC fraction 48 or 54, washed and then added to microwells containing CTL-DC#1 at an effector-to-target cell ratio of 2:1. To each of these wells were then added either media, unlabeled T2 cells or T2 cells preloaded with either the $p53_{264-272}$ or $MDM\text{-}2_{53-61}$ peptides at a cold:hot T2 ratio of 10:1. After 4 h incubation at 37°C, supernatants were harvested, radioactive counts (cpm) determined and the mean percentage (± SD) of cold target inhibition calculated from triplicate determinations. The data presented are from one assay of two performed which yielded comparable results.

In vitro generation of MDM-2-specific CD8+ T cells from HLA-A2+ normal donors and patients with HNC

Synthetic MDM-2 peptides were pulsed onto DCs and used to drive the *in vitro* expansion of autologous effector CD8+ T cells isolated from 5 normal HLA-A2+ donors and 4 HLA-A2+ patients with HNC. As shown in Figure 6A, most of the normal donors exhibited responses against both of the MDM-2 peptides, with a general bias towards recognition of the MDM-2_{53-61} epitope. In marked contrast, a preliminary analysis of *in vitro* response to these peptides in HLA-A2+ patients with HNC revealed minimal or no reactivity (Figure 6B).

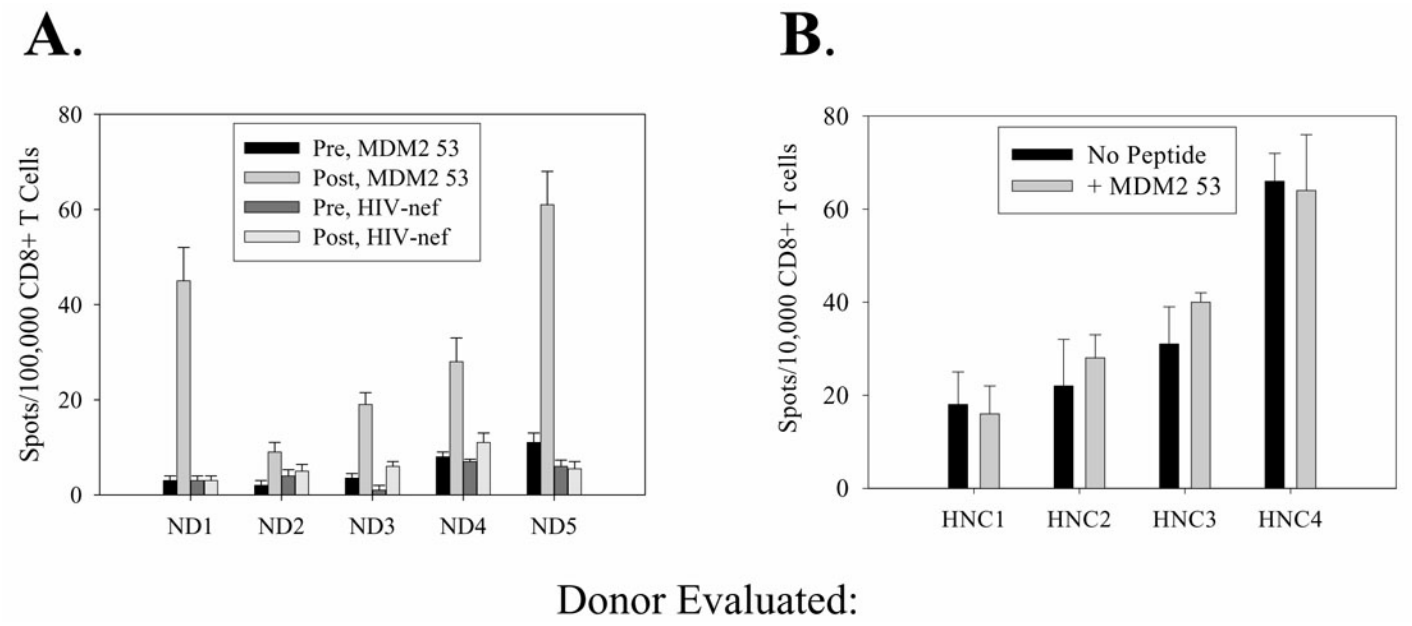

Figure 6. MDM-2_{53-61} is immunogenic in HLA-A2+ normal donors but not in patients with HNC. DCs were prepared from the peripheral blood of 5 normal donors (panel A) and 4 patients with HNC (panel B) as outlined in Materials and Methods, pulsed with the MDM-2_{53-61} peptide for 2 h at 37°C, prior to washing and addition to autologous responder T cells at a T:dendritic cell ratio of 50:1. A second identical DC/peptide restimulation was provided one week later and CD8+ T cells were analyzed for specific reactivity one week after this secondary boost in IFN-gamma ELISPOT assays against T2 control cells or T2 cells pulsed with the indicated peptides. HIV-nef peptide was included as a negative control. All determinations were performed in triplicate, with results reported as the mean ± SD.

CD8+ T cell clones were generated from a normal HLA-A2+ donor in limiting dilution cultures stimulated with the MDM-2_{53-61} peptide. These T cell clones recognized HLA-A2+, MDM-2-overexpressing target cell lines, including the HNC PCI-13 and PCI-1 and the MEL526 melanoma lines, but not the HLA-A2+, MDM-2 ^{low} gastric carcinoma, T2, or HLA-A2-negative SCC-74 HNC cell lines (Figure 7A). This clone also recognized T2 targets pulsed with the synthetic MDM-2 epitope or HPLC-fractionated PCI-13-derived peptides (i.e., fraction 48) (Figure 7B). Unpulsed T2 target cells or T2 cells pulsed with PCI-13 peptides present in HPLC fraction 52 (containing the p53 epitope as suggested in Figure 5) were not recognized by this clone. In all cases, specific recognition of these target cell lines by the CD8+ T cell clone was blocked by addition of anti-HLA-A2 mAbs (Fig 7A and data not shown).

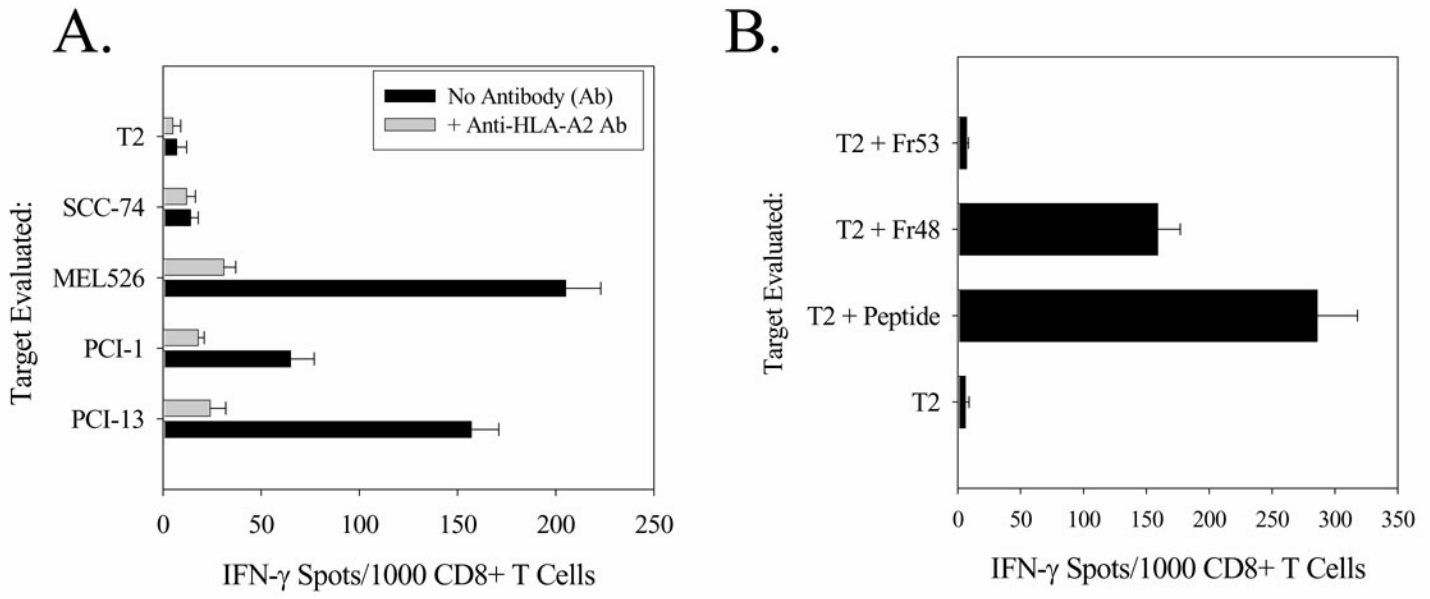

Figure 7. A CD8+ T cell clone specific for the MDM-2_{53-61} peptide recognizes HLA-A2+ tumor target cell lines that overexpress MDM-2 and natural peptides contained in HPLC fraction 48 derived from the PCI-13 HNC line. Clone 1A1 was harvested from a well seeded at 0.3 cell/well in limiting dilution culture from a normal HLA-A2+ donor stimulated *in vitro* with MDM-2_{53-61} peptide-pulsed autologous DCs. After expanding the clone to sufficient numbers, it was analyzed against a range of target cell lines that vary in their constitutive levels of HLA-A2 and MDM-2 protein using the IFN-gamma ELISPOT assay (panel A). The PCI-13 and PCI-1 HNC and the MEL526 melanoma lines are HLA-A2+ and overexpress the MDM-2 protein (as determined by Western Blot and IHC analyses, Figure 3 and data not shown). The T2 cell line is HLA-A2+, but fails to overexpress MDM-2, while the SCC-74 HNC cell line is HLA-A2-negative. T cell recognition was blocked by addition of the MA2.1 anti-HLA-A2 mAb. The CD8+ T cell clone also selectively recognized T2 targets loaded with the synthetic MDM-2_{53-61} peptide or peptides found in HPLC fraction 48 derived from PCI-13 target cells (panel B). Data are reported as the mean ± SD from triplicate determinations and are representative of two assays performed.

Discussion

The current study was initially undertaken to compare two methods of *in vitro* vaccination using physiologically-relevant but distinct antigen-presenting cells (tumor vs. DCs) for their ability to promote the generation of HNC-reactive CTLs and to define the nature of tumor-associated epitopes recognized by CTLs induced as a result of such *in vitro* sensitization (IVS). We chose to evaluate the HLA-A2+ PCI-13 SCC cell line as both the tumor stimulator in MLTC-type vaccinations and the source of lysate for the loading of autologous DCs. This allowed us to compare the effectiveness of the relevant antigens in the different vaccines and to serve as a potential model for human clinical trials, where autologous tumor may not be available or accessible for vaccine preparation. Overall, both types of *in vitro* vaccines were successful in promoting the expansion of HLA-A2 restricted CTL, capable of recognizing HNC cell lines. However, autologous DCs loaded with PCI-13 lysate yielded slightly higher frequencies of specific CTLs in bulk responder populations after 4 weeks of stimulation. The DC-based approach was also more often successful in promoting the qualitative activation of anti-HNC reactive CTLs.

A detailed analysis of one of the CTL lines generated on lysate-pulsed DCs (CTL-DC#1, CTL-DC#2 and CTL-DC#3) revealed that fully 6-10% of CD8+ T cells were reactive against HLA-A2+ HNC cell lines (such as PCI-13 and PCI-1) within the bulk HLA-A2+ responder population. The HLA-A2-restricted repertoire in the CTL-DC lines capable of recognizing HNC targets appeared to be focused on 5-6 predominant shared epitopes, based on the HPLC-based fingerprinting of these CTL lines. The HLA-2-restricted repertoire of specificities did vary between donors (i.e. CTL-DC#1 vs. CTL-DC#2), although three shared epitopes did appear to be recognized by both responders.

It was gratifying to observe that two of the subsequently analyzed synthetic peptides (MDM-2_{53-61} and $p53_{264-272}$) co-eluted with natural epitopes found in HPLC fractions 48 and 54, respectively. Furthermore, the frequency of effector cells in the CTL-DC#1 line capable of recognizing the corresponding synthetic as well as natural peptides is quite similar and the synthetic sequences effectively cold-target inhibited the natural epitopes in these fractions, providing additional evidence that the natural and synthetic peptide species are most likely related. Lastly, cloned CD8+ T cells that recognize the synthetic MDM-2_{53-61} peptide also recognize both HLA-A2+ HNC and melanoma target cell lines and natural PCI-13-derived peptides contained in HPLC fraction 48 providing, perhaps the strongest support for the relatedness of these immunogens. While these data remain circumstantial in nature and do not substantiate the absolute identity of the unknown peptides in HPLC fractions 48 and 54, they indicate that a targeted prospective mass spectrometric analysis of these fractions for sequences identical to or containing known MDM-2 and p53-derived HLA-A2-binding peptides is likely to be successful. Based on our interesting results for MDM-2, we are currently in the process of screening additional predicted HLA-A2 binding peptides derived from p53-dependent proteins (coordinately overexpressed as a result of p53 mutation and accumulation) (29) for biologically relevant CTL epitopes.

We have demonstrated the ability to drive the expansion of effector CTLs with semi-allogeneic (i.e., HLA-A2 matched) tumor cells or isolated HLA-A2+ tumor-derived peptides that exhibit potent specificity for HLA-A2+ relevant HNC targets. Indeed, when HLA-A2-restricted peptides (MDM-2_{48-57} and MDM-2_{53-61}) were used for IVS on DCs with autologous PBMCs obtained from normal donors, specific CD8+ T cells were readily generated in most donors. In theory, this provides a logical and feasible method of IVS and/or *in vitro* expansion of anti-tumor CTLs in/from any HLA-A2+ individual. Such a method would be particularly attractive for HLA-A2+ patients with HNC tumors for whom the autologous tumor tissue is either unavailable or unusable for these purposes. We expect that for HLA-A2+ patients, who represent half of the Caucasian patient population (30), with HNC, the MDM-2 (identified in this study) and p53 epitopes (identified in this and previous studies) (16, 23, 28, 29) could provide the possibility for constructing therapeutic DC/synthetic peptide-based vaccines.

However, it was disappointing, although not altogether unexpected, that we could not generate MDM-2_{53-61}-specific CD8+ T cells from patients with HNC using the same protocol that was able to successfully do so from normal donors. We have previously published similar observations for the $p53_{264-272}$ peptide (23), where an MHC/p53 peptide tetramer analysis of HNC patients indicated that the lack of responsiveness most likely involved low frequencies of circulating T cells able to recognize this "self" epitope. It is possible that T cells mediating surveillance against such "overexpressed" epitopes are deleted or hyporesponsive to stimulation due to defects in TCR signaling in HNC patients (3, 4, 5). We are currently evaluating the conditions under which crosspriming promoted by DC-based (lysate or synthetic peptide) vaccines may best overcome the immunological unresponsiveness of HNC patients in order to develop an effective therapeutic regimen for these individuals.

Abbreviations

HNC, head and neck cancer

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Materials and methods

Lymphocytes, cell lines and HLA typing

Peripheral blood mononuclear cells were obtained from leukapheresis products of healthy HLA-A2+ platelet donors and isolated from heparinized blood by Ficoll-Hypaque centrifugation. Washed PBMCs were used for CTL generation or DC preparation, and the remaining cells were cryopreserved and stored at -80°C. Squamous cell HNC lines and non-squamous cell carcinoma lines were generated and maintained in our laboratory, as previously described (31, 32). MHC class I and II typing of PBMCs from healthy donors was performed by the Tissue Typing Laboratory, University of Pittsburgh Medical Center. For tumor cell lines, MHC class I and II antigens were determined by using peripheral blood lymphocytes at the time tumor cell lines were established or by DNA analysis of tumor cell lines performed by the Tissue Typing Laboratory. Table 2 lists the HLA allotypes of the tumor and lymphocyte lines used in the experiments described in this report.

Generation and culture of CTLs

In all experiments, dendritic cells were used as antigen presenting cells. Tumor-associated protein for the loading of DCs was generated from 1% trifluoroacetic acid lysates of PCI-13 cells, as previously outlined (19). Isolated PCI-13 protein was then coated onto Dynabeads (Dynal Biotech, Lake Success NY; M-280 Tosylactivated beads; 50-100 µg protein from 10^7 PCI-13 cells with 5 µl of beads) overnight at 4°C, prior to feeding these coated beads to DCs for 2 h at 37°C. To start the T-cell culture, cryopreserved non-adherent lymphocytes, obtained after plastic-adherent PBMCs were separated for DC generation, were thawed into AIM-V medium containing 5% human AB serum but no cytokines. Cells were adjusted to a cell density of 5 x 10^6/ml in T25 flasks, and irradiated, Dynabead/protein-fed DCs were then added as stimulator cells (50:1 T:DC cell ratio). After a period of one week, the following cytokines were added to the T-cell cultures: IL-1beta (0.2 ng/ml), IL-2 (10 IU/ml) and IL-7 (0.2 ng/ml). Seven days later, a second stimulation (identical to the first) was performed. For all subsequent stimulation cycles, irradiated tumor cells (PCI-13) were used, as described above. Generally, 4 cycles (unless otherwise noted) of stimulation with tumor cells were necessary for the generation of effector cells exhibiting HLA-A2-restricted reactivity against PCI-13 target cells. For the maintenance of established CTL lines, restimulation was supported by AIM-V medium containing only IL-2 (20 IU/ml) and IL-7 (0.2 ng/ml) in addition to 5% (v/v) human AB serum.

For the induction of peptide-specific T cells, autologous day 7 DCs were pulsed with peptides at a 10 µM concentration for 2 h at 37°C, washed, and added to bulk PBMC T-cell responders at a T:DC ratio of 50:1. A second identical DC/peptide restimulation was performed one week later. The following cytokines were added to T-cell cultures during the secondary stimulation culture period: IL-1beta (0.2 ng/ml), IL-2 (10 IU/ml) and IL-7 (0.2 ng/ml). CD8+ T cells were isolated by MACS™ (Miltenyi, Biotec Inc., Auburn, CA.) separation one week later and analyzed in IFN-gamma ELISPOT assays.

Dendritic cell generation

To generate autologous DCs, HLA-A2+ PBMCs obtained from a leukopack were suspended in AIM-V medium at a cell density of 1 x 10^7/ml in T162 flasks (COSTAR, Cambridge, MA) as described previously (33). After 1 h incubation in 5% CO_2 in air at 37°C, non-adherent cells were decanted. Residual non-adherent cells and platelets were removed by 5 vigorous washes with serum-free RPMI-1640 medium prior to addition of AIM-V medium containing 1000 IU/ml of IL-4 and 1000 IU/ml of GM-CSF (kindly provided by Schering-Plough Research Institute, Kenilworth NJ) to the adherent PBMCs. The cultures were incubated in 5% CO_2 in air at 37°C for 7 days. On day 7, DCs were harvested, irradiated (30 Gy) and pulsed with a protein/bead preparation as outlined above.

Cytotoxicity assays

Four-hour ^{51}Cr-release assays were performed in triplicate as previously described (31) using various tumor cell lines as targets. The percent specific lysis was calculated according to the formula:

Percent specific lysis = (experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm) x 100

For functional blocking studies, tumor cells were pre-incubated with anti-MHC class I (W6/32 or MA2.1) or II (anti-DR, L243) mAbs for 30 min at 40°C before being used as targets in cytotoxicity assays. Lymphocytes were pre-incubated with azide-free, purified mAbs to CD3, CD4 or CD8 surface markers (Becton Dickinson, Mountainview, CA) for 30 min at 4°C prior to use in cytotoxicity assays. For cold target inhibition assays, unlabeled cold targets were used at a cold:hot target ratio of 10:1.

ELISPOT assays

To determine the frequency of T cells capable of responding to a specific stimulus by secretion of IFN-gamma, ELISPOT assays were performed as previously described (33, 34). On the day of the assay, total CD3+ or CD8+ T cells, positively selected using specific MACS™ beads (Miltenyi), were placed in the wells of 96-well plates. Ten thousand T2 cells (or tumor cells), peptides and blocking or control mAbs were added per well and the plates were incubated for 20 h at 37°C. The number of spots in an individual well was quantitated by the Zeiss AutoImager using KS software as previously described (33). All determinations were performed in triplicate with the mean ± SD reported for each data set for the indicated number of cultured unseparated T cells or MACS™-purified CD8+ T cells placed in the well. For blocking studies, 5 µg of Protein-G (Sigma, St. Louis, MO) purified blocking mAb (purified from the culture supernatant of hybridomas purchased from the American Type Culture Collection, Rockville, MD) was added per well.

Limiting dilution assays

Limiting dilution assays were performed as previously described (33). Lymphocytes were seeded in 96-well plates at 10, 3 and 1 cell/well with 5 x 10^3 irradiated (100 Gy) PCI-13 cells used as stimulators and 2-5 x 10^4 irradiated (30 Gy) allogeneic PBMCs obtained from a healthy donor used as feeder cells. Culture medium was AIM-V containing 5% (v/v) heat-inactivated human AB serum, IL-1beta (0.2 ng/ml), IL-2 (10 IU/ml) and IL-7 (0.2 ng/ml). Two to three weeks later, wells containing proliferating lymphocytes were counted in order to determine the frequency of cytolytic T lymphocyte precursors. In some cases, cloned CD8+ T cells were expanded and analyzed for reactivity against tumor and peptide-pulsed target cell lines.

Flow cytometry

PBMCs or cultured lymphocytes were stained with fluorescein- or phycoerythrin-labeled monoclonal Abs to surface antigens CD3, CD8, CD4, HLA-A2 (Becton Dickinson, Mountainview, CA) and examined by flow cytometry as described earlier (12). Isotype control antibodies were included in all experiments.

Western blotting and immunohistology

Cells (5-10 x 10^6) were analyzed for specific protein expression in Western blots using the following anti-human Abs reactive against: p53 (murine mAb clone D0-7, Pharmingen, San Diego, CA) and MDM-2 (murine mAb clone SMP14, Pharmingen). Cell pellets were lysed using 200 µl of 1% NP-40 in PBS containing protease inhibitors (Complete, Boehringer Mannheim, Indianapolis, IN) for 1 h on ice. After centrifugation at 13,500 x *g* for 30

minutes, the supernatant was mixed 1:1 with SDS-PAGE running buffer and proteins separated on 10% PAGE gels, prior to electroblotting onto nitrocellulose membranes (Millipore, Bedford, MA). Blots were imaged on Kodak X-Omat Blue XB-1 film (NEN Life Science Products, Boston, MA) using horseradish peroxidase-conjugated goat anti-mouse Ig (Biorad, Hercules, CA) and the ECL chemiluminescence detection kit (NEN Life Science Products).

For immunostaining, PCI-13 cells or human keratinocytes established from skin explants in short-term cultures using keratinocyte growth mediums (LifeTechnologies, Grand Island, NY) were cytocentrifuged onto glass slides. Following fixation, immunoperoxidase staining was performed using anti-MDM-2 Abs from Castra Laboratories (Burlington, CA), anti-p53 Abs from BioGenex, (San Ramon, CA) and the appropriate IgG isotype controls.

HPLC fractionation of HNC-derived peptides

HNC cell lines (PCI-13, PCI-1 and SCC-74) were grown in cell factories (Nunc™, purchased from Fisher Scientific, Pittsburgh, PA) until they reached a density of approximately 5 x 10^8 cells/factory. MHC class I-bound peptides were then extracted by brief acid elution as previously described (20, 21, 22). After recovery of the peptide-containing supernatant, the residual viable cells were washed and re-cultured overnight in complete medium, prior to a second round of acid stripping. The combined peptide material from two procedures was equivalent to a harvest from 10^9 PCI-13 cells. After concentration and lyophilization, peptides were resolved on reverse-phase HPLC as previously described (20, 21, 22). Peptide-containing fractions were lyophilized to remove organic solvent and then resuspended in 100 µl of PBS, prior to storage at -80°C until use in ELISPOT assays.

Peptide selection and synthesis

The protein sequence of the human MDM-2 gene product (Accession No. NP_002383) was obtained from RefSeq (NCBI) and subsequently inspected for potential HLA-A2-binding peptides using a computer algorithm (26) designed by Dr. Ken Parker (27). The top two MDM-2 scoring peptides were selected (Table 3) and synthesized by the Peptide Synthesis Facility (University of Pittsburgh Cancer Institute, Shared Resource). All peptides were HPLC-purified and were >95% pure based on their HPLC profile and the MS/MS mass spectrometric analysis performed by the UPCI Protein Sequencing Facility (Shared Resource).

Mass spectrometric sequencing

HPLC fractions were lyophilized to near dryness and resuspended in 5 µl of 0.1 M acetic acid. One microliter of this material was loaded onto a microcapillary C_{18} HPLC column (150 mm × 75 µm i.d.). Peptides were eluted with a linear gradient (0-80% buffer B, 20 min) using a buffer system consisting of 0.1 M acetic acid in ddH_2O (buffer A) and 0.1 M acetic acid in 100% acetonitrile. Effective flow rates for the nanospray probe (200 nl/min) were achieved by using the Rainin HPLC system previously described equipped with an Accurate microflow processor (LC Packings, San Francisco, CA) for flow splitting. The nanospray probe was operated at a voltage differential of +3.2 keV. The source temperature was maintained at 30°C. Mass spectra were obtained by scanning from 400-1,500 every 2.6 sec and summing individual spectra on a Fisons Quattro II (Fisons, Loughborough, U.K.). Collision-induced dissociation was performed by selecting mass ions of interest and scanning at 500 atomic mass units/sec using 133 mPa of Ar in the collision chamber.

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