Cancer Immunity, Vol. 5, p. 5 (9 March 2005)
Submitted: 9 September 2004. Accepted: 24 December 2004.
Communicated by: HF Oettgen

CANCER
IMMUNITY
Article

Characterization of antigen-specific CD8+ T lymphocyte responses in skin and peripheral blood following intradermal peptide vaccination

Qiyuan Chen[1], Heather Jackson[1], Mark Shackleton[1,2*], Phillip Parente[1,2], Wendie Hopkins[1], Sue Sturrock[2], Duncan MacGregor[2], Eugene Maraskovsky[1], Tsin Yee Tai[1], Nektaria Dimopoulos[1], Kelly-Anne Masterman[1], Tina Luke[1], Ian D. Davis[1,2], Weisan Chen[1], and Jonathan Cebon[1,2]

[1]*Ludwig Institute for Cancer Research, Heidelberg, Victoria, Australia*
[2]*Austin Hospital, Heidelberg, Victoria, Australia*
[*]*Present address: The Walter and Eliza Hall Institute of Medical Research (WEHI) , Victoria, Australia*

Keywords: clinical trial, melanoma, vaccination, peptides, immunological monitoring

Immune responses to cancer vaccines are commonly tested by measuring cutaneous reactions to intradermal (i.d.) antigen. When well-characterized peptide epitopes are injected i.d., infiltrates of CD4+ and CD8+ T lymphocytes are frequently seen. In this study, we have further characterized T cells derived from vaccine-infiltrating lymphocyte (VIL) responses. We found that the infiltrates capable of producing IFN-gamma and cytolytic activity could recognize vaccine peptide, as well as antigen-positive melanoma cells. We studied antigen-specific T cell responses from VILs and peripheral blood in 10 patients who participated in a clinical trial. All patients received systemic Flt3 ligand (20 μg/kg/d) and i.d. peptides: Three NY-ESO-1 peptides, SLLMWITQCFL (157-167), SLLMWITQC (157-165), QLSLLMWIT (155-163); tyrosinase internal peptide YMDGTMSQV (368-376); Melan-A/MART-1 analogue peptide ELAGIGILTV (26-35, E27L substitution); and influenza matrix peptide GILGFVFTL (58-66). In 54 paired VIL and peripheral blood analyses, a good correlation was found between responses in skin and in blood. These cells could be rapidly expanded in a short-term assay and thus appear to be memory T cells. The demonstrated presence of antigen-specific T cells at vaccination sites validates this method of assessing the immune response to i.d. vaccines.

Introduction

One of the major challenges facing the field of tumour immunology is to identify the most useful assays for evaluating immune responses against cancer antigens. For patients participating in clinical trials with cancer vaccines, defined antigens (commonly peptides or, more recently, proteins) can be administered by i.d. or s.c. injections. These antigens stimulate immune responses to antigens that are present on cancer cells, with the expectation that such responses might modify the natural history of the disease. Indeed, a number of clinical trials have demonstrated that objective tumour regressions can occur in the setting of such vaccinations, presumably due to the induction of antigen-specific T cell responses (1, 2, 3). However, robust clinical responses or durable clinical benefit are not consistently seen, and concerted efforts are being made to optimize vaccination strategies. In the absence of reproducible clinical endpoints, surrogate endpoints are commonly employed to evaluate the effectiveness of one vaccination strategy or another. A variety of immune surrogates have been described (4), including the quantification of CD8+ T cells in peripheral blood and the measurement of cutaneous inflammatory responses to injected antigen.

Although cutaneous reactions to injected antigens have frequently been measured and cellular infiltrates characterized phenotypically (5, 6, 7), it is also important to establish that the immune cells in these lesions are specific for the antigen in question. Techniques for evaluating these cells *in situ* are hampered by the limited methodologies suitable for demonstrating the specificity of these cells. Additionally, these techniques cannot determine the capacity to further respond to specific antigens. Peripheral blood is most commonly drawn to assay antigen-specific T cells or their precursors, and these assays are helpful when positive. However, to date there has been no consistent correlation between clinical regression and circulating T cell responses. There are numerous examples of tumour regression following vaccination in which it has not been possible to quantify significant numbers of circulating effectors (8, 9, 10). In such cases, it can be assumed that effectors have localized and possibly proliferated at sites where antigen is present, even if such cells cannot be identified at high frequencies in the peripheral blood. To assay these cells, tumour biopsies can be performed and, where this is practical, TILs can be characterized (11, 12, 13). In those cases where tumour is not accessible, alternative approaches are required. In this study, we report on the use of biopsies taken at the sites of antigen injection. This is a readily accessible source of effectors that have localized to tissue sites having a specific antigen. T cells extracted from such sites have been characterized to determine antigen specificity. The results of these studies have been related to two more commonly employed assays: (i) ELISPOT assays performed on peripheral blood and the measurement of induration and (ii) erythema reactions at the site of antigen injection (DTH reactions).

Results

DTH responses to i.d. peptides are characterized by CD4+ and CD8+ T cell infiltrates

The details of the clinical, haematological, and immune outcomes of this trial are described in detail elsewhere (14). A subset of patients was evaluated in this study. The characterization of the cutaneous reactions to peptide antigens is shown in Table 1. Of these 60 i.d. peptide injection sites, 21 were scored as having DTH reactions when the cutoff for reactivity was prospectively defined as 5 mm or more of palpable induration. In addition, 7/60 showed redness but a lesser degree of induration, and so did not qualify as positive reactions (Table 1). Responses were seen to all peptides except the tyrosinase peptide YMDGTMSQV, although some peptides elicited responses more frequently than others. As expected, responses to the influenza peptide occurred commonly; however responses to the NY-ESO-1 SLLMWITQCFL (157-167) peptide were also seen in 6/10 patients. Figure 1 shows the macroscopic and histological appearance of DTH sites in a representative patient. A dense perivascular infiltrate of CD3+ and CD4+ T lymphocytes was seen with a substantial population of CD8+ cells.

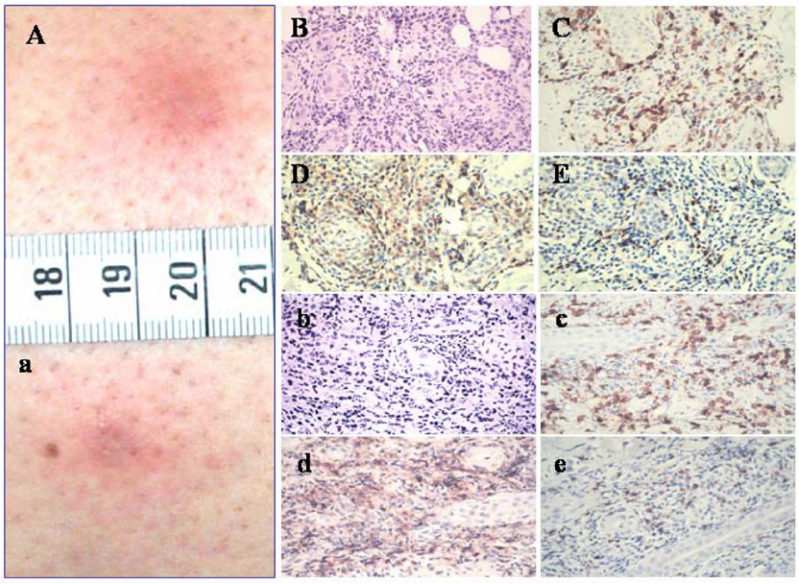
Table 1

Diameter of induration and redness of DTH lesions[a]

Patient	Peptide											
	$ESO1_{157-167}$		$ESO1_{157-165}$		$ESO1_{155-163}$		Tyrosinase		MelanA/MART-1 analogue		Influenza	
	SLLMWITQCFL		SLLMWITQC		QLSLLMWIT		YMDGTMSQV		ELAGIGILTV		GILGFVFTL	
	Red	Ind	Red	Ind	Red	Ind	Red	Ind	Red	Ind	Red	Ind
1	32	23	22	20	13	15	0	0	6	2	38	31
2	10	0	0	0	0	0	4	0	6	0	9	0
3	0	0	0	0	0	0	0	0	0	0	16	16
4	4	7	6	10	6	10	4	1	5	2	26	7
5	3	0	5	2	2	0	0	0	0	0	0	0
6	7	11	5	6	4	5	4	2	2	0	9	10
7	4	12	3	4	2	3	3	2	4	6	7	27
8	20	20	0	0	20	15	0	0	0	0	62	30
9	45	16	0	0	0	0	0	0	4	3	6	2
10	2	0	0	0	0	0	0	0	0	0	25	16
Positive		**6/10**		**3/10**		**4/10**		**0/10**		**1/10**		**7/10**

[a]Red: Diameter of redness (mm). Ind: Diameter of induration (mm). A positive reaction was scored for all DTH lesions showing induration ≥5 mm. Shaded areas show positive reactions.

Figure 1



Representative example of a DTH response to vaccination with NY-ESO-$1_{157-167}$ (A, B, C, D, E) or influenza matrix peptide (a, b, c, d, e). Macroscopic appearance (A, a). Hematoxylin/eosin staining (B, b). Immunostaining of CD3 (C, c), CD4 (D, d), and CD8 (E, e).

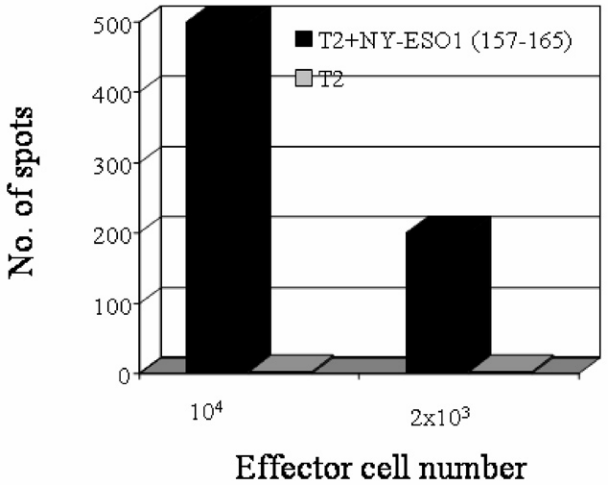
Infiltrating lymphocytes at the site of vaccination show antigen specificity

In order to further characterize T cells expanded from DTH biopsies, we generated an NY-ESO-$1_{157\text{-}165}$-specific T cell line derived from a DTH site of a vaccine recipient known to have had a good DTH response. The cells were presensitized with vaccine peptide (SLLMWITQC) *in vitro* and then cultured as described in the Materials and Methods section. These cells were then further characterized by different functional assays, including ELISPOT, intracellular IFN-gamma production, cytolytic activity, TCRs on tetramer staining and its TCR beta variable region (Vbeta) usage. It was found that these, like CTLs, could produce IFN-gamma and kill target cells *in vitro*. The TCRs could be bound by tetramer. They could not only recognize peptide-pulsed targets, but could also recognize melanoma cell lines in an MHC class I-restricted manner. This recognition could be enhanced by IFN-gamma induction of antigen expression on tumour cells. With the TCR Vbeta analysis, we demonstrated that antigen-specific CTLs derived from VILs and PBLs used at least one Vbeta subunit in common (vbeta16), indicating that these VILs and the peripheral blood cells were derived from the same population (Figures 2, 3, and 4).

Antigen-specific CD8+ T cell responses occur both at vaccination sites and in peripheral blood

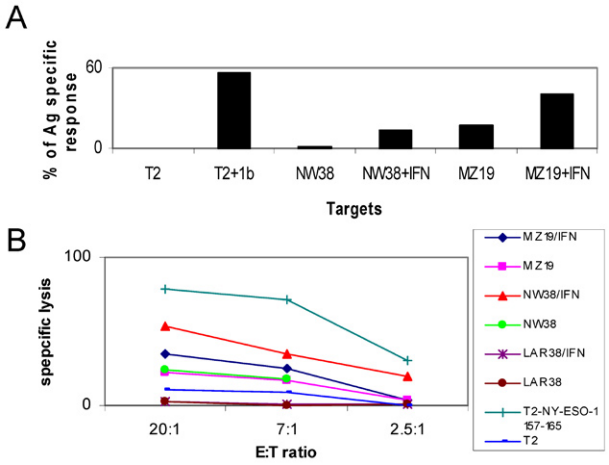
We next compared the detection of antigen-specific T cells in skin biopsy sites and in peripheral blood to determine the concordance between effectors at sites of injected antigen and in the circulation. The clinical trial protocol did not specify simultaneous sampling of DTH lesions and blood: skin biopsies were performed 2 d after peptide injection, whereas blood was drawn after 14 d. Data are shown in Table 2. Paired data are available for 54 biopsies. A biopsy sample was arbitrarily scored positive for antigen-specific T cells if more than 40 spots were seen in the ELISPOT assay. Positive and concordant results were seen in the blood and skin of 6/54 paired samples, including 3 samples for NY-ESO-$1_{157\text{-}167}$. In 6 cases (3 Melan-A/MART-1 samples and 3 influenza matrix samples), antigen-specific CD8+ T cells were found in blood but not in cutaneous sites. For both of these antigens, it is well known that antigen-specific T cells can circulate without prior vaccination. In one NY-ESO-$1_{157\text{-}165}$ sample, antigen-specific cells were isolated from the biopsy, but not from blood. Tyrosinase-specific responses were not detected from skin or blood in any sample. In contrast, influenza responses were common: 6/10 blood samples and 3/10 tissue samples were positive.

Figure 2



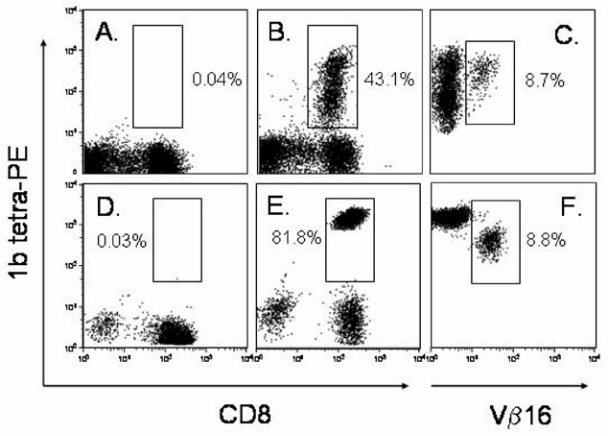
ELISPOT assay showing the detection of IFN-gamma-producing T cells specific for the NY-ESO-$1_{157\text{-}165}$ peptide. Background spots are not subtracted.

Figure 3



Recognition of natural antigen NY-ESO-1 on tumour cell lines. In this assay, both NW-38 and MZ-Mel-19 (MZ19) are HLA-A2-positive and NY-ESO-1-positive, whereas LAR38 is HLA-A2-positive but NY-ESO-1-negative. T cells derived from VILs could recognise T2 cells pulsed with NY-ESO-$1_{157\text{-}165}$ peptides, as well as cultured melanoma cell lines. This recognition could be enhanced by IFN-gamma induction of tumour cells. Antigen-specific response was measured by intracellular IFN-gamma production (A) and by cytolytic capacity (B).

Figure 4



TCR vbeta16 usage of NY-ESO-$1_{157\text{-}165}$-specific T cells in VILs and PBLs. In this assay, T cells were assessed by triple staining, CyChrome-conjugated anti-CD8 Ab, FITC-conjugated vbeta16 Ab, and PE-conjugated tetramer (NY-ESO-$1_{157\text{-}165}$-specific). (A), (B), and (C) correspond to a T cell line derived from PBLs. (D), (E), and (F) correspond to a T cell line derived from VILs. The gated populations represent cells which are both vbeta16 and tetramer positive.

Table 2

Relationship between antigen-specific T cells in peripheral blood (14 d after vaccination) and at vaccination sites (2 d after vaccination)[a]

Patient	Peptide											
	$ESO1_{157-167}$		$ESO1_{157-165}$		$ESO1_{155-163}$		Tyrosinase		MelanA/MART-1 analogue		Influenza	
	SLLMWITQCFL		SLLMWITQC		QLSLLMWIT		YMDGTMSQV		ELAGIGILTV		GILGFVFTL	
	PB	VIL	PB	VIL	PB	VIL	PB	VIL	PB	VIL	PB	VIL
1	145/23	98/5	34/7	94/9	0	0	26/5	0	30/9	0	64/4	44/3
2	2	4	0	0	4	0	3	0	7	2	20	0
3	4	7	7	11	0	3	0	3	61/12	7	111/20	33/12
4	1	12	0	2	0	0	0	13	26/4	0	19	28/5
5	0	1	0	0	1	1	0	2	82/4	0	35/10	0
6	0	0	0	1	0	1	0	0	0	2	72/37	2
7	0	4	0	0	0	0	4	0	58/4	0	180/10	165/4
8	65/5	230/30	Nt	Nt	Nt	0	Nt	0	Nt	0	260/20	250/0
9	95/5	188/8	Nt	0	Nt	2	0	0	0	0	155/5	0
10	0	8	0	0	0	0	0	0	16	0	170/10	30/2
Positive	**3/10**	**3/10**	**0/8**	**1/9**	**0/8**	**0/10**	**0/9**	**0/10**	**3/9**	**0/10**	**6/10**	**3/10**

[a]A positive reaction was scored when more than 40 spots were counted. Shaded areas show positive reactions. The number of spots/background is shown for all positive samples. Abbreviation: PB, peripheral blood; VIL, vaccine infiltrating lymphocytes; Nt, not tested.

Discussion

Vaccine strategies for the treatment of cancer can induce immune responses; however these may not correlate with clinical benefit. Successful vaccine-based immunotherapy of cancer requires (i) induction of an adequate immune response, (ii) appropriate localization of immune effectors to cancer sites, and (iii) susceptibility of cancer cells to immune-mediated killing. While assays for circulating T cells can identify the presence of an immune response, they provide little information about the capacity of these cells to localize to sites where antigen is present in tissue. Furthermore, a negative blood test does not necessarily indicate that a vaccine did not generate effector cells, but simply that such cells are not evident in the circulation at the time of venesection. It therefore becomes essential to evaluate methods for detecting antigen-specific cells in tissue sites.

We have shown that CD8+ and CD4+ T cells are recruited to sites of i.d. peptide vaccination. The CD8+ T cells expanded from these sites were frequently specific for the vaccinated peptide. As shown in Figure 3, they could also recognize natural tumour antigen, produce IFN-gamma, and kill in a cytotoxicity assay. De Vries *et al*. studied two patients following vaccination with peptide-loaded DCs and made similar observations. They also found that cells derived from DTH reactions could kill tumour cells and thus could provide useful insights into the specificity and effector capacity of vaccine-induced immune responses (15).

Demonstrating a good correlation between the detection of antigen-specific cells in both blood and skin reactions gives us greater confidence that DTH reactions are true measures of antigen-specific immunity. Consequently, unless nonspecific responses occur to diluents or contaminants, a positive DTH response can be thought to be antigen-specific with greater confidence even without these corroborating assays. Furthermore, in some cases this response appeared more sensitive than the other assays of cellular immune function.

We and others have previously demonstrated that objective clinical responses to vaccination may not correlate well with a detectable immune response (8,9). Although immune monitoring can provide useful immunological endpoints for evaluating the efficacy of a vaccine, such surrogate endpoints require validation by demonstrating a correlation between immune and clinical endpoints. Numerous methods for immunological monitoring have been used. We have shown that immune effectors can be harvested from sites of antigen injection in a standardized manner. These cells represent a population that has the capacity to leave the circulation and target a specific antigen in tissue sites. When antigen-specific effectors are found in the skin, they are also usually found in the circulation: however, cells found in the tissues may represent a more relevant population. The one exception to this general observation was seen in a single patient for the HLA-A2-restricted 9-mer NY-ESO-$1_{157-165}$, in which a skin response but no blood response was seen (Table 2). Larger numbers of patients must be evaluated following vaccination with this peptide in order to determine if this is more commonly observed. If so, monitoring responses to this antigen will need to take this observation into account. We also found that antigen-specific CD8+ T cells could be isolated more frequently from patients with larger DTH reactions. This is consistent with the observations of others (16).

In some patients, circulating T cells specific for Melan-A/MART-1 or influenza matrix peptide were found in the absence of skin reactions (Table 2). Circulating cells specific for these antigens had previously been reported following vaccination (2), but have also been found in nonvaccinated subjects, where cross-recognition (17) or prior antigen exposure (in the case of influenza) can result in positive assays. The failure to detect a response in some patients may be because these responses were preexisting and not induced by vaccination. Additionally, there may have been a poor response to injected antigen in the skin of these patients because of its rapid disappearance (related to peptide solubility and stability after injection) or because of failed recognition with the use of a peptide analogue.

In summary, this study reveals the involvement of antigen-specific T lymphocytes in the inflammatory cutaneous responses that can follow i.d. peptide injection. Since these T cells reflect a population of effector cells that is capable of localizing to sites of antigen within tissues, their detection represents an important surrogate immune endpoint for assessing responses to cancer vaccines.

Abbreviations

VIL, vaccine-infiltrating lymphocyte

Acknowledgements

We thank Amgen/Immunex for supplying the Flt3 ligand. Ian D. Davis was supported in part by an Australia National Health and Medical Research Council Career Development award.

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Materials and methods

Subjects and vaccination program

Subjects participated in clinical trial LUD 97-012, conducted at the Ludwig Institute for Cancer Research at the Austin Hospital in Melbourne, Australia. The Austin Health Human Research Ethics Committee approved the clinical trial, and all participants provided written informed consent. All patients had either evaluable melanoma or resected stage II, III, or IV melanoma at high risk of relapse, were HLA-A2+, and expressed one or more melanoma antigens by immunohistochemistry or RT-PCR. The peptide-based vaccine consisted of five defined melanoma-associated peptides, including three overlapping NY-ESO-1 peptides, SLLMWITQCFL (157-167), SLLMWITQC (157-165), and QLSLLMWIT (155-163); tyrosinase internal peptide YMDGTMSQV (368-376); and Melan-A/MART-1 analogue peptide ELAGIGILTV (26-35, E27L substitution). An influenza matrix peptide, GILGFVFTL (58-66), was used as a positive control. Flt3 ligand, a haematopoietic growth factor, was used as an adjuvant. Three cycles of Flt3 ligand (20 μg/kg) were administered daily s.c. for 14 d, followed by a 14 d rest period. Peptides were injected i.d. on day 8 and day 15 of each cycle. Each peptide was injected at a separate site. Three NY-ESO-1 peptides were provided at 100 μg in 330 μL DMSO, Melan-A/MART-1 and influenza matrix peptides at 100 μg in 100 μL PBS with 30% of DMSO, and tyrosinase internal peptide at 100 μg in 100 μL PBS with 10% DMSO. The Melan A/MART-1, tyrosinase, and influenza peptides were administered i.d. at 100 μg/dose. Poor solubility of the NY-ESO-1 peptides required formulation in DMSO, so each NY-ESO-1 peptide was diluted 1:3 using sterile saline solution prior to administration. A total of 100 μL (33 μg) was injected for each peptide.

As part of the monitoring, DTH responses and antigen-specific T cell responses from peripheral blood were evaluated. Cutaneous reactions at sites of peptide injections were measured 48 h after vaccination both by palpating skin induration and by measuring erythema. Reactions were considered to be clinically positive if the skin induration exceeded 5 mm in diameter and was associated with erythema (Table 1).

Immunohistochemistry

Serial 4-μm formalin-fixed paraffin sections of the DTH sites were mounted on SuperFrost® Plus slides and dried overnight at 37°C. Following dewaxing in xylene and rehydration through alcohol, microwave antigen retrieval in 1 mM EDTA buffer at pH 8.0 was performed for antibodies to CD3, CD4, and CD8 (BD Biosciences, San Jose, CA, USA). A 700-watt NEC N702EP microwave was used on the high setting to heat the slides for 10 min, followed by a 20-min cooling period at room temperature. Sections for CD8 staining were also subjected to antigen retrieval in the microwave using 0.01 M citrate buffer, pH 6.0.

Immunohistochemistry was performed using the Vectastain Elite Universal ABC kit purchased from Vector Laboratories (Burlingame, CA, USA). Endogenous biotin activity was quenched by sequential application of egg white and skim milk. All incubations were performed at room temperature using the Shandon Sequenza immunostainer. The chromogen 3-amino-9-ethyl-carbazole (Sigma Chemical, St. Louis, MO, USA) was used and slides were counterstained with Mayer's hematoxylin (Amber Scientific, WA, Australia). Application of CrystalMount (Biomeda, CA, USA) preceded dehydration and mounting in DePeX (BDH, Poole, UK).

Cell culture

The T2 cell line and the human melanoma cell lines were all cultured in RPMI 1640 medium supplemented with 10% FCS (CSL, Victoria, Australia), 5 x 10^{-5} M 2-ME, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Dr. Old kindly provided the MZ-Mel-19 cell line; the NW38 cell line was a gift from Dr. Knuth of Zurich, Switzerland. These two cell lines are HLA-A2+ and NY-ESO-1+. The melanoma cell line LAR38 was established in our laboratory. Antigen expression by the melanoma cells was assessed by both immunohistochemistry and RT-PCR (18).

VIL preparation

Punch biopsy (4 mm diameter) specimens were obtained from peptide injection sites. Half of each sample was finely minced with a surgical scalpel in RPMI 1640 medium supplemented with 10% FCS (CSL, Victoria, Australia). The cell suspension was placed in a 96-well, round-bottomed plate in 200 μL/well of RPMI medium, supplemented with 10% heat-inactivated human AB serum (Sigma Chemical, St. Louis, MO, USA), 10 IU/mL human recombinant IL-2 (Cetus, Emeryville, CA, USA), and 10 ng/mL human recombinant IL-7 (Pepro Tech, Rochy Hill, NJ, USA). The samples were presensitized with irradiated (3000 rads) autologous PBLs pulsed with the corresponding peptide (10 μg/mL) for 9 to 14 d before being analysed. Yields from the culture ranged from 10^3-10^5 cells per biopsy. CTL lines used in this study were NY-ESO-$1_{157\text{-}165}$-specific and were maintained *in vitro* by periodic peptide restimulation.

Antigen-specific CD8+ T cells from peripheral blood

Peripheral blood was drawn 2 wk after peptide injections; mononuclear cells were separated by Ficoll-density gradient centrifugation and cryopreserved. To assay for antigen-specific CD8 T cells, 1 x 10^7 frozen peripheral blood leukocytes were thawed and CD8+ T cells were enriched using anti-CD8 beads (Dynabeads, Dynal Biotech, Oslo, Norway) according to the manufacturer's instruction. CD8-negative cells were used as APCs. They were resuspended in serum-free medium, pulsed with 10 μg/mL peptide, and incubated at 37°C for 1 h. These cells were then irradiated at 3000 rads and washed twice before being added to the culture. CD8+ T cells were cocultured with peptide-pulsed APCs at a ratio of 1:1 (10^6:10^6) in RPMI supplemented with 10% heat-inactivated human AB serum in a 96-well, flat-bottomed plate. IL-2 (10 IU/mL; Cetus, Emeryville, CA, USA) was added on day 2 and the cells were further incubated at 37°C, 5% CO_2 for 7 d.

IFN-gamma ELISPOT

The method used was adapted from a previously published method (1). In brief, ELISPOT plates (Millipore Multiscreen, MAHA S45, Bedford, MA, USA) were coated with 5 μg/mL antihuman IFN-gamma mAb (CSL, Victoria, Australia) overnight at 4°C. The plate was blocked with 200 μL of PBS supplemented with 10% FCS. T2 cells were pulsed with 10 μg/mL peptide in serum-free RPMI for 1 h, and excess peptide was removed by washing once. Effector cells were cocultured with peptide-pulsed T2 cells or T2 cells alone (control). After 16-18 h in culture, the cells were removed and 200 μL distilled water was added to ensure lysis of any remaining viable cells. After 1 h at room temperature, the water was removed. Captured IFN-gamma was detected at sites of secretion by incubating with rabbit polyclonal antihuman IFN-gamma Ab (CSL, Victoria,

Australia) at room temperature for 2 h. After extensive washing with PBS containing 0.05% Tween 20, AEC substrates (Sigma Chemical, St. Louis, MO, USA) were added to each well. Colour development lasted for 6-8 min and was stopped by washing in tap water.

Intracellular IFN-gamma detection by flow cytometry

For intracellular IFN-gamma detection, cultured VIL cells were tested for their antigen-specific responses against vaccinated peptide and control peptide. In brief, T2 cells were pulsed with NY-ESO-1 peptide or a control peptide not used for vaccination, MAGE-3 peptide FLWGPRALV (271-279), for 1 h at room temperature. Excessive peptide was removed by washing, and target cells were cocultured with effector cells in the presence of Brefeldin A 10 μg/ml (Sigma Chemical, St. Louis, MO, USA). After 4 h of stimulation, the culture was washed once with PBS and stained with CyChrome-conjugated mouse antihuman CD8 mAb (BD Pharmingen, San Diego, CA, USA) at 4°C for 30 min. After one wash, the permeabilization solution, 1% paraformaldehyde, was added to the cell pellet which was then incubated at room temperature for 20 min. IFN-gamma was then stained by incubation with FITC-conjugated mouse antihuman IFN-gamma Ab (BD Pharmingen, San Diego, CA, USA), and cells were analysed by flow cytometry using the Flowjo program (19).

Tetramer assay and TCR analysis

A NY-ESO-$1_{157-165}$ tetramer containing the analogue substituted C165A peptide SLLMWITQA was a gift from the Lausanne Branch of the Ludwig Institute, Switzerland. Anti-TRC Vbeta was purchased from Serotec (Raleigh, NC, USA). In brief, the VILs or PBLs were first stained with PE-coupled HLA-A2/NY-ESO-$1_{157-165}$ tetramer in 50 μL of PBS plus 2% FCS at 4°C for 30 min, CyChrome-conjugated antihuman CD8 Ab (BD Biosciences, San Jose, CA, USA) and FITC-conjugated anti-Vbeta Ab were then added and the cells further cultured at 4°C for another 30 min. The cells were washed once and analysed in a FACSCalibur™ (BD Biosciences, San Jose, CA, USA).

IFN-gamma induction of tumour cell lines

In order to enhance tumour antigen expression, melanoma cell lines were precultured with 100 ng/ml recombinant human IFN-gamma (Pepro Tech, NJ, USA) for 48 h before being used as targets. The melanoma cells were simultaneously phenotyped for their MHC class I expression by w6/32 Ab (Sigma Chemical, St. Louis, MO, USA).

^{51}Cr release assay

In brief, effector cells were washed and seeded in 96-well, V-bottomed microtitre plates (Costar, MA, USA) using three-fold dilutions at different effector-to-target (E:T) ratios. Targets (10^6) were labelled with 150 μCi ^{51}Cr (Amersham Biosciences, Amersham, UK) in 0.5 ml RPMI 1640 for 1 h at 37°C. After two washes, ^{51}Cr-labelled T2 cells were resuspended in 1 ml RPMI 1640 medium and pulsed with 1 μg/ml peptide. The cells were then incubated at 37°C for another 30 min. After one wash, the targets were cocultured with effector cells for 4 h. At the end of the incubation period, 50 ul supernatants were removed for counting in a gamma counter (TopCount NXT, Packard Bioscience, CT, USA). The results were expressed as the percentage of specific cytotoxicity, calculated as 100 x [test-spontaneous ^{51}Cr release (%)]/[maximum-spontaneous ^{51}Cr release (%)].

Contact

Address correspondence to:

Jonathan Cebon
Ludwig Institute for Cancer Research
Austin Hospital
Heidelberg, VIC 3084
Australia
Tel.: + 61 3 9496-5462
Fax: + 61 3 9457-6698
E-mail: Jonathan.cebon@ludwig.edu.au