

Human Dendritic Cells Genetically Engineered to Express a Melanoma Polyepitope DNA Vaccine Induce Multiple Cytotoxic T-Cell Responses¹

Steven Gerard Smith, Poulam Manubhai Patel, Joanne Porte, Peter John Selby, and Andrew Mark Jackson²

Applied Immunology Group, Imperial Cancer Research Fund, Cancer Medicine Research Unit, St. James's University Hospital, Leeds, LS9 7TF, United Kingdom

ABSTRACT

Purpose: To assess the therapeutic potential of a melanoma polyepitope vaccine in human cells. Polyepitope DNA vaccines encoding T-cell epitopes have been demonstrated in murine systems to generate multiple cytotoxic T-cell responses to different antigens. Here, for the first time we demonstrate the ability of a melanoma polyepitope to stimulate lymphocytes from normal human donors to simultaneously generate multiple antigen-specific responses.

Experimental Design: Human dendritic cells (DC), transduced with a melanoma-polyepitope cDNA, were used to activate autologous lymphocytes from naïve donors as an *in vitro* model of DNA vaccination. Lymphocytes were primed with polyepitope or mock-transduced DC, boosted with peptide, then measured for antigen-specific cytotoxicity.

Results: Lymphocytes primed with polyepitope-transduced DC and boosted with peptide generated multiple cytotoxic responses. By contrast lymphocytes primed with mock-transfected DCs and boosted with peptide gave no specific cytotoxicity. However, when lymphocytes were repeatedly stimulated with polyepitope-transduced DCs immunodominance was seen with CTLs being generated to only one epitope, MART_{27–35}.

Conclusions: We show in a human system that a melanoma polyepitope primes CTL to multiple epitopes. However, repeated stimulation by the polyepitope restricts the response to only the MART1 epitope. Thus, although polyepitope vaccines are an effective way of priming multiple naïve T-cell responses, continual boosting with polyepitope

vaccines may, as a result of immunodominance, restrict the CTL. These findings have important implications for the use of DNA polyepitope vaccines in cancer immunotherapy.

INTRODUCTION

The discovery of melanoma-associated antigens has resulted in a proliferation of clinical trials based on antigen- and epitope-specific immunization against cancer. Protocols have used direct injection of peptide epitopes with adjuvant (1) or alternatively, DCs³ pulsed with peptide epitopes and readministered to patients (2). Although peptide injection is logistically easier, adoptive transfer of *ex vivo* pulsed DCs may provide more potent activation signals to CTLs, avoiding their deletion attributable to presentation by nonprofessional adenomatous polyposis coli (3). This is largely because DCs possess high levels of MHC and costimulatory molecules necessary for potent activation of helper and cytotoxic T cells (4). Although CTL responses are elicited in patients immunized by both methods, these are often not associated with significant clinical regression of tumors, and many mechanisms have been described by which tumors can evade immune responses (5). Down-regulated expression of antigen by tumor cells is one of the more effective ways of evading an epitope-restricted CTL response. Strategies that use several antigens can circumvent this, and the polyepitope approach (encoding multiple MHC class I-restricted epitopes as one cDNA) is one such example (6). Studies in mice revealed that when virally vectored polyepitopes were administered, CTL responses were elicited despite the lack of natural flanking sequences (7–9). Therefore, this approach allows epitopes from different antigens to be administered simultaneously, reducing the antigen loss evasion mechanisms of tumors. Polyepitopes also negate the need to vaccinate with whole, potentially oncogenic DNA sequences (10). In addition polyepitopes can be delivered as plasmid DNA and injected directly without the need for viral vectors, and can incorporate signal sequences that improve processing or include MHC class II-restricted helper epitopes (11–13).

To date, work with polyepitope vaccines for cancer has focused on murine models. In human leukocyte antigen-A2 transgenic mice, responses vary from 50% to 100% of epitopes (14–16). However, the immune repertoire and antigen-processing machinery in man varies significantly from that of mice. Importantly, as many melanoma antigens are “self-antigens”

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² To whom requests for reprints should be addressed, at Applied Immunology Group, Imperial Cancer Research Fund, Cancer Medicine Research Unit, St. James's Hospital, Leeds, LS9 7TF, United Kingdom. Phone: 44-113-206-4463; Fax: 44-113-242-9886; E-mail: Andrew.Jackson@icrf.icnet.uk.

³ The abbreviations used are: DC, dendritic cell; MD-DC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; MART, melanoma antigen recognized by T-cells; MAGE, melanoma antigen; PBL, peripheral blood lymphocyte; TNF, tumor necrosis factor; IL, interleukin; ICRF, Imperial Cancer Research Fund.

tolerance may prevent the successful use of these in vaccines in man, despite apparent efficacy in mice. Cultured DCs have been used to test human immune responses *in vitro* and, when modified with peptide, DNA, or recombinant virus, induce CTL responses from naïve lymphocytes (17). Responses to a single epitope from influenza virus were described when a polyepitope incorporating this epitope was tested in retrovirally transduced DCs (18). However, many individuals have been exposed previously to influenza, and so this may not be an ideal model for a tumor epitope (19).

We designed a polyepitope to encode multiple melanoma-associated epitopes restricted to HLA-A1 and HLA-A2. When human lymphocytes were primed with polyepitope-transduced DCs and boosted with peptide, multiple CTL responses were seen. However, repeated boosting to polyepitope-transduced DCs resulted in a single dominant CTL response to an epitope from the MART-1/Melan-A melanoma antigen. This may have implications for the scheduling of polyepitope DNA vaccination and DC-based vaccines in humans.

MATERIALS AND METHODS

Polyepitope Construction. The polyepitope sequence (296 bp) was synthesized using four oligonucleotides (Iain Goldsmith, ICRF Oligonucleotide Synthesis Service) that overlapped by 20 bp and together covered the full polyepitope sequence. The cDNA was assembled by PCR using the splicing by overlap extension technique (9). Briefly, adjacent primers were dimerized in separate PCR reactions after which primer dimers were spliced together to generate the full-length sequence. Products from the oligonucleotide splicing by overlap extension steps were used as templates for a final PCR amplification reaction using 20-bp terminal primers. Gel-purified polyepitope cDNA (termed poly-MEL) was restricted using *Bam*HI and *Eco*RI and cloned into the pVAX1 expression vector (Invitrogen). Constructs were sequenced to verify their fidelity.

Transfection of COS7 Cells. Before transfection (24 h), COS7 cells were seeded into six-well plates (Costar; 2.5×10^5 cells/well) in DMEM (Life Technologies, Inc.) supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin (Life Technologies, Inc.). Transfections were carried out using 2.5 μ g of pVAX1 vector or pVAX-poly-MEL with 15 μ l of DOTAP reagent (Boehringer Mannheim) for immunofluorescence or with 12 μ l of LipofectAMINE reagent (Life Technologies, Inc.) for Western blotting in 1 ml of medium for 5 h. After transfection (24 h), cells were trypsinized, transferred to glass slides, and cultured for an additional 48 h. Alternatively, transfectants for lysis and Western blotting were cultured continuously in six-well plates for 72 h.

Indirect Fluorescence Confocal Microscopy. After transfection (72 h), cells were fixed with 3.7% formaldehyde/PBS and then permeabilized in 0.1% Triton-X-100/PBS. Permeabilized cells were probed with anti-pk antibody (Serotec) in PBS and detected with Alexa-Fluor 488-conjugated goat antimouse antibody (Molecular Probes Inc.) and confocal fluorescence microscopy (Leica TCS-SP). Cell cytoskeleton and nuclei were stained with Phalloidin conjugated to Alexa-Fluor 594

(Molecular Probes Inc.) and 4',6-diamidino-2-phenylindole, respectively, and slides mounted in Mowiol 4–88 (Calbiochem).

Western Blotting. Transfected cells were resuspended in disruption buffer [5% glycerol, 50 mM Tris-HCl (pH 7.0), 2% SDS, 5% 2-mercaptoethanol, and 0.25% bromphenol blue] and stored at -20°C . After boiling for 5 min, samples were electrophoresed on a 16% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham) using the Novex Xcell II blotting apparatus. After blocking in Tris-buffered saline with 0.1% Tween plus 10% skimmed milk the membrane was incubated with anti-pk antibody. Bound antibody was detected using biotinylated rabbit antimouse (Dako), horseradish peroxidase-conjugated streptavidin (Dako), and enhanced chemiluminescence (Amersham).

Recombinant Adenovirus Containing Poly-MEL. Recombinant adenovirus was synthesized using the AdEasy system (20). The pAdEasy1 adenoviral backbone vector, the pAdTrack-CMV shuttle plasmid, and the *Escherichia coli* strain BJ5183 were gifts from Dr. Tong-Chuan He (John Hopkins Oncology Center, Baltimore, MD). The 293 packaging cell line was obtained from ICRF Central Cell Services and maintained in DMEM supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin (Life Technologies, Inc.).

Poly-MEL was subcloned into the pAdTrack-CMV shuttle plasmid using the *Bam*HI and *Eco*RI restriction sites. Electrocompetant BJ5183 were then cotransformed with 1 μ g of *Pme*I linearized pAdTrack-CMV/poly-MEL and 0.1 μ g of supercoiled pAdEasy1. Plasmids obtained from ampicillin-resistant colonies were screened for recombinant pAd-poly-MEL vectors, which were subsequently amplified in DH5 α *E. coli* (Life Technologies, Inc.). Control virus was generated using “empty” pAdTrack-CMV vectors. Cells (293) were transfected with 4 μ g of *Pac*I linearized pAd-poly-MEL or pAd-empty and 20 μ l of LipofectAMINE reagent/25 cm² flask (Costar). After 7 days, cells were harvested and lysed by four cycles of freeze/thaw/vortexing to obtain viral supernatants. Adenovirus was amplified by repeated infection of 293 cells, and virus supernatants were stored at -20°C . The inclusion of the green fluorescent protein gene in pAdTrack vectors was used to estimate viral titers by infecting 293 cells and counting green fluorescent cells by fluorescence microscopy after 24 h.

HLA-A2 Typing. Aliquots of PBMC from buffy coats of healthy donors were tested with the anti-HLA A2 antibody BB7.2 (ICRF Research monoclonal antibody service), the binding of which was detected by FITC-conjugated goat antimouse (Dako) and flow cytometry.

Generation of MD-DCs. Buffy coats obtained from healthy blood donors were provided by the National Blood Service (Seacroft Hospital, Leeds, United Kingdom). PBMC were isolated by centrifugation of diluted buffy coats through Lymphoprep (Nycomed). After washing, cells were transferred to six-well plates (Costar) at 2×10^7 PBMC/well in RPMI 1640 with 2% heat-inactivated fetal calf serum, L-glutamine, and penicillin/streptomycin, and incubated for 1.5 h at 37°C . The nonadherent fraction were harvested and frozen, and the adherent cells were cultured for 6 days in 3 ml/well of DC medium (RPMI 1640/10% HI-FCS supplemented with 800 units/ml granulocyte macrophage colony-stimulating factor (Leucomax; Schering Plough) and 500 units/ml IL-4 (R&D Systems).

Medium (1 ml) was replaced with fresh medium plus cytokines on day 3. On day 6 immature MD-DCs were harvested as nonadherent cells.

Adenoviral Transduction of MD-DCs. Adenovirus infection was carried out by incubation of 10^6 immature MD-DCs in 1 ml of adenoviral supernatant (multiplicity of infection = 100) for 2 h at 37°C. Transduced DCs were washed and transferred to a 24-well plate at 10^6 cells/ml of DC medium including 1000 units/ml of TNF α (R&D Systems) for 24 h to induce maturation.

In Vitro Generation of CTL Responses. TNF α matured, 7-day-old, transduced MD-DCs were irradiated (3000 rads) and mixed with autologous PBLs that comprised the nonadherent fraction of PBMC used to generate MD-DCs. Cells were combined to give 10^5 MD-DCs and 2×10^6 PBL/ml of CTL medium [RPMI 1640, 7.5% heat-inactivated human serum type AB (Sigma Chemical Co.), L-glutamine, penicillin/streptomycin, HEPES, nonessential amino acids, sodium pyruvate (Life Technologies, Inc.), and 200 μ M 2-mercaptoethanol (Sigma Chemical Co.)] plus 5 ng/ml of IL-7 (R&D systems). Cells were distributed into 24-well plates (Costar) at 1 ml/well and cultured at 37°C. After 3 days and as required thereafter, cultures were fed with CTL medium plus 5 ng/ml IL-7. IL-2 (Proleukin) was added at this stage to a final concentration of 20 IU/ml. On day 7 and weekly thereafter, responding PBL were restimulated with autologous PBMC pulsed separately with peptide epitopes (Dr. Nicola O'Reilly, ICRF Peptide Synthesis Laboratory) corresponding to the A2-restricted epitopes from poly-MEL. For pulsing, PBMC were resuspended at 10^6 cells/ml in serum-free RPMI 1640 with L-glutamine and penicillin/streptomycin. Peptide was added to a final concentration of 50 μ g/ml after which pulsing was carried out at 37°C with occasional agitation of cell suspensions. After 4 h, PBMC were washed, irradiated (3000 rads), and cocultured with responder PBL at a ratio of 1:1 in CTL medium with IL-7. Alternatively PBL primed in week 1 with adenovirally transduced MD-DCs were restimulated weekly with fresh poly-MEL transduced MD-DCs as described above.

Cytotoxicity Assays. Responding PBL were tested for specific cytotoxicity in chromium release assays. T2 cells (174xCEM.T2 hybridoma, transporter associated with antigen presentation-1-, and transporter associated with antigen presentation-2-deficient; American Type Culture Collection) were simultaneously labeled with of [51 Cr]sodium chromate (Amersham) at 100 μ Ci/ 10^6 cells and peptide-pulsed with 10 μ g/ml of each melanoma epitope, influenza matrix epitope₍₅₈₋₆₆₎ or with no epitope. After three washes, target cells were mixed with responder PBL so the killing by each population was tested against targets loaded with relevant, control and no peptide. "Cold" K562 cells (no MHC expression and sensitive to natural killer cell-mediated lysis) were included at $50 \times$ the target cell number to inhibit nonspecific lysis. Assays (4-h) were carried out in 96-well plates with 5000 targets/well in a final volume of 200 μ l RPMI 1640 with 10% FCS. All of the E:T ratios were tested in triplicate. Spontaneous release was determined in medium alone and maximal release in 1% Triton-X-100 (Sigma Chemical Co.). Chromium release was assayed by liquid scintillation using a 1450 MicrobetaJet (Wallac), and percentage-specific cyto-

Table 1 Tumor antigens and related epitopes included in the poly-MEL polyepitope

Tumor antigen ^a (epitope position)	Epitope sequence (HLA restriction)	Score ^b
gp100 (154–162)	KTWGGYVQV (A2)	316
gp100 (280–288)	YLEPGPVTA (A2)	1
MAGE-1 (161–169)	EADPTGHSY (A1)	250
MAGE-3 (161–169)	EVDPIGHLY (A1)	250
MAGE-3 (271–279)	FLWGPRALV (A2)	2655
Melan-A/MART-1 (27–35)	AAGIGILTV (A2)	2
Tyrosinase (1–9)	MLLAVLYCL (A2)	309
Tyrosinase (368–376)	YMDGTMSQV (A2)	213

^a Refs. 22–27.

^b Estimated half time of disassociation, Ref. 21.

toxicity was calculated using the following formula: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] \times 100.

Staining with Fluorogenic HLA-Tetramer. Cells were stained in phosphate buffered saline with phycoerythrin-labeled MART-1 tetramer at 37°C for 20 min, washed at room temperature, and incubated on ice with antibodies to CD8 (CD8-PerCP; BD Biosciences).

Statistics and Epitope Binding Predictions. Analysis of cytotoxicity data were performed using two-tailed Student's *t* tests assuming equal variance. Epitope binding predictions were obtained using an algorithm based on Parker *et al.* (21).

RESULTS

Polyepitope: Poly-MEL. Eight epitopes described previously, known to be recognized on the surface of melanoma cells by CTL, were chosen for inclusion in poly-MEL (Table 1). Only epitopes restricted to HLA-A1 or HLA-A2 were included, because these two alleles are common in melanoma-prone populations. Epitopes were directly linked without flanking sequences in the poly-MEL sequence, which included a Kozac consensus start sequence, the SV5-pk antibody tag, a stop codon, and restriction sites to facilitate future modifications (Fig. 1A). Human optimized codon usage was used, and unavoidable internal start codons were positioned to minimize initiation of translation.

When transfected COS-7 cells were probed using anti-pk antibody, staining of the cytoplasm was seen in pVAX1/poly-MEL-transfected cells (Fig. 1B). In addition, Western blotting revealed a specific protein corresponding with the predicted mass, absent in mock-transfected cells (Fig. 1C). Thus, recombinant pol-MEL protein is synthesized by transfected cells. This is in agreement with the results of Hanke *et al.* (12), who similarly used the pk tag to successfully label an HIV polyepitope. The poly-MEL sequence was then expressed in an adenoviral system for efficient delivery to MD-DC. We have shown this system previously to potently transduce DC without perturbing their maturation or function (28). Despite Western blotting, confocal microscopy, and flow cytometry, we have not detected the polyepitope in adenoviral-transduced DC.

Polyepitope-transduced DC Prime Multiple CTL Responses. To measure induction of T-cell responses in a human system, *in vitro* stimulation of lymphocytes from four

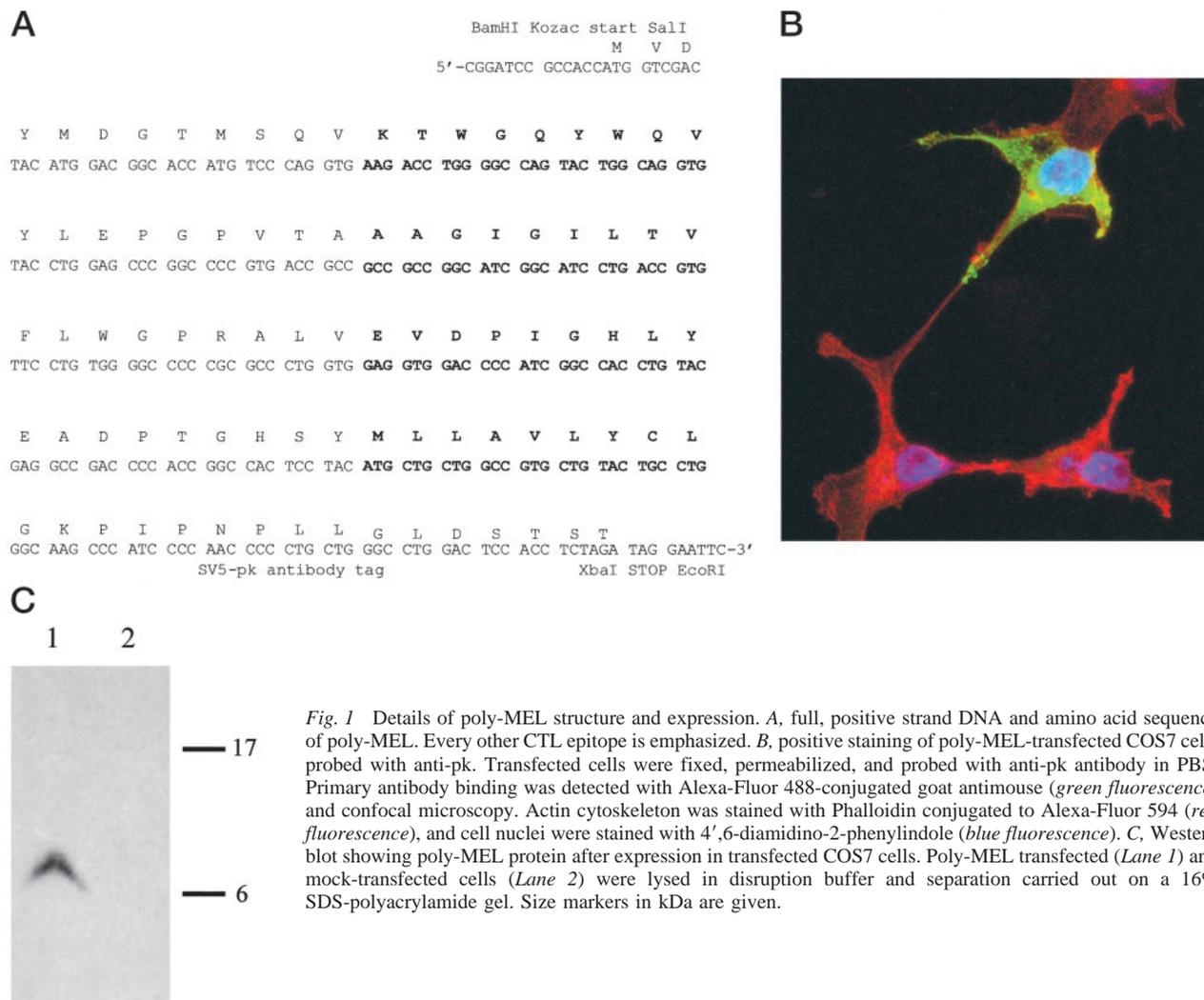


Fig. 1 Details of poly-MEL structure and expression. **A**, full, positive strand DNA and amino acid sequence of poly-MEL. Every other CTL epitope is emphasized. **B**, positive staining of poly-MEL-transfected COS7 cells probed with anti-pk. Transfected cells were fixed, permeabilized, and probed with anti-pk antibody in PBS. Primary antibody binding was detected with Alexa-Fluor 488-conjugated goat antimouse (*green fluorescence*) and confocal microscopy. Actin cytoskeleton was stained with Phalloidin conjugated to Alexa-Fluor 594 (*red fluorescence*), and cell nuclei were stained with 4',6-diamidino-2-phenylindole (*blue fluorescence*). **C**, Western blot showing poly-MEL protein after expression in transfected COS7 cells. Poly-MEL transfected (*Lane 1*) and mock-transfected cells (*Lane 2*) were lysed in disruption buffer and separation carried out on a 16% SDS-polyacrylamide gel. Size markers in kDa are given.

HLA-A2+ donors was carried out. Poly-MEL or mock-transduced DC were used to prime autologous lymphocytes for 1 week. Transduced DC displayed high levels of CD40, CD80, and MHC class II, which were up-regulated after exposure to TNF α (data not shown), confirming that DC were not adversely affected by adenoviral transduction. After priming, responder lymphocytes were “boosted” separately in week 2 and thereafter with autologous PBMC pulsed with peptide. Cytotoxicity assay demonstrated that multiple CTL responses were generated from lymphocytes primed with poly-MEL-transduced DC (Fig. 2). CTL for all six of the HLA-A2-restricted epitopes were elicited from 1 donor (Fig. 2B). Responses to 5 of 6 epitopes were seen in two donors (Fig. 2, C and D), whereas in donor A, were generated to three epitopes. No cytotoxicity was seen toward influenza matrix peptide loaded T2 targets or of unloaded T2 cells. By contrast lymphocytes stimulated by mock-transfected DCs and restimulated with peptide generated no epitope specific CTLs. (Fig. 2, mock prime). These results demonstrate that all six of the HLA-A2-restricted epitopes from poly-MEL

could be processed by human DCs and presented to autologous T lymphocytes from naive donors. Furthermore, this allowed priming of the corresponding CTL responses from PBL, which were measurable after expansion in the presence of peptide epitopes.

The frequency of MART-1-specific T cells was determined by staining unstimulated and primed/boosted lymphocytes with tetrameric-HLA-A2/MART-1. Two of four donors had detectable MART-1-specific CTL before stimulation. Activation and expansion of CTL by poly-MEL resulted in a significant increase in the proportion of CD8+ cells stained with the MART-1 tetramer (Fig. 3). This was observed for all four of the donors.

Restricted CTL Induction by Repeated Stimulation with Polyepitope. To determine whether poly-MEL could prime and boost multiple CTL responses in human lymphocytes, autologous lymphocytes were repeatedly stimulated with poly-epitope or mock-transduced DC. In all four of the donors, after three rounds of restimulation, specific killing was only seen of MART-1₂₇₋₂₅ loaded targets. (Fig. 4). Even after five rounds of

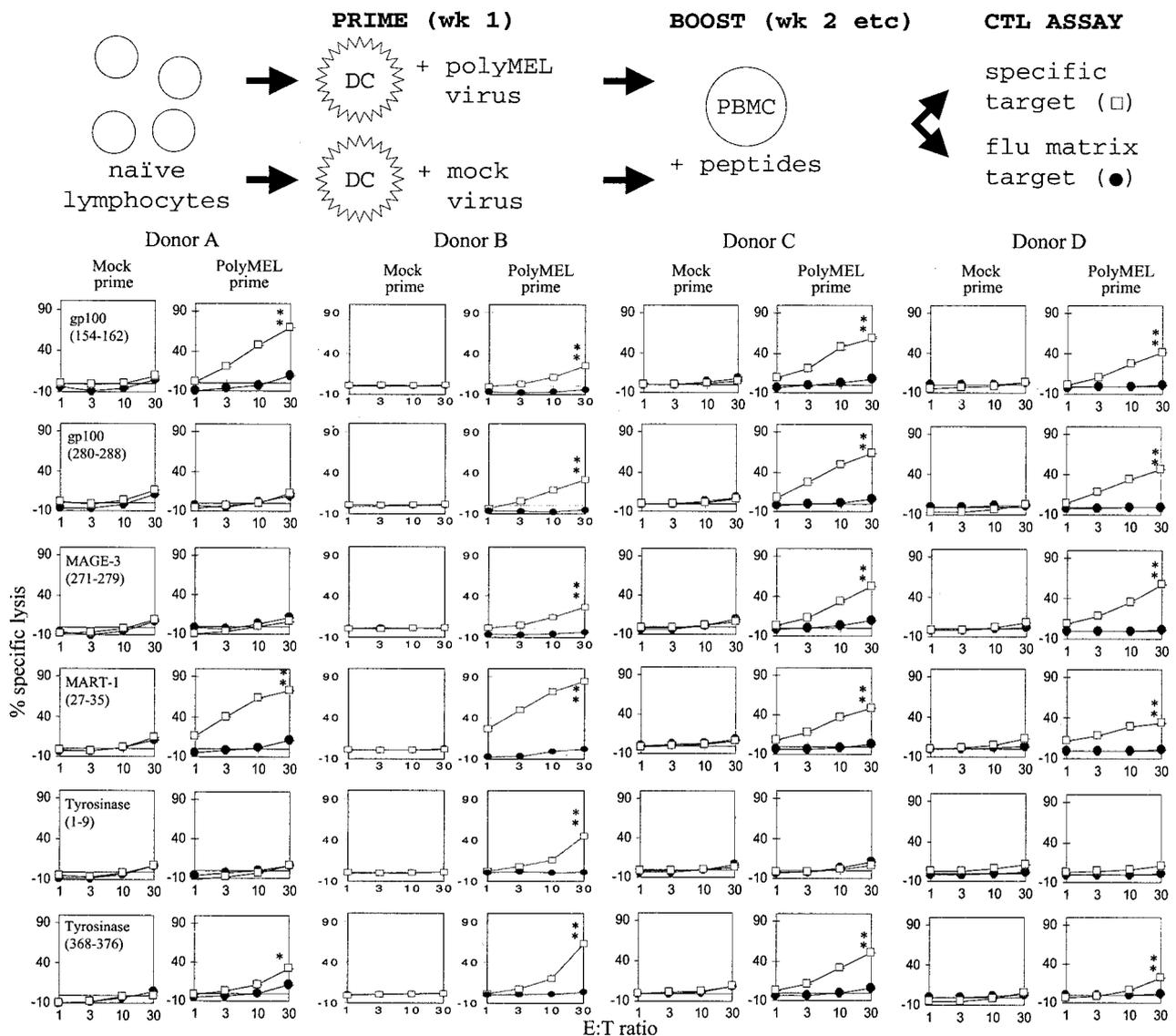


Fig. 2 Multiple CTL responses induced by poly-MEL. CTL responses after MD-DC priming followed by restimulation with autologous, peptide-pulsed PBMC as shown in cartoon form. Responder PBL were primed in week 1 with either poly-MEL or mock-transduced MD-DC as indicated. From week 2 onwards, polyepitope and mock-primed cells were split and restimulated using PBMC pulsed with the peptide epitope indicated in the first graph of each row. Killing of 51 chromium-labeled T2 cells was tested for each responder population after three restimulations. Targets were pulsed with either the specific peptide epitope indicated for each row (□) or with flu matrix (58–66) epitope (●). Each point represents the mean and SD of triplicate experiments. Significant differences in cytotoxicity against specific and control targets at the 30:1 E:T ratio are indicated (** $P < 0.001$, * $P < 0.005$). Representative results from four donors are given.

stimulation with polyepitope-transduced DC, it was not possible to generate any other CTL specificities (data not shown). These findings illustrate that despite the ability to prime CTL to all of the HLA-A2-restricted epitopes, repeated stimulation with polyepitope-transduced DC induced CTL responses to 1 of 6 epitopes, namely MART-1_{27–35}.

DISCUSSION

This study sought to address the therapeutic potential of a polyepitope vaccine designed to induce multiple CTL responses against melanoma antigens. These included the tu-

mor-specific antigens MAGE-1 and MAGE-3 as well as differentiation antigens gp100, tyrosinase, and MART-1. Expression of tumor-specific and differentiation antigens has been described for a large proportion of patients with melanoma with heterogeneous expression of gp100 and MART-1 but consistent tyrosinase expression on all of the melanoma-associated metastatic lesions examined (29). In addition, MAGE-1 and MAGE-3 are expressed in 30–70% patients with metastatic melanoma illustrating the potential value of multivalent vaccines. We have shown that our polyepitope is translated into protein and can prime responses to the major-

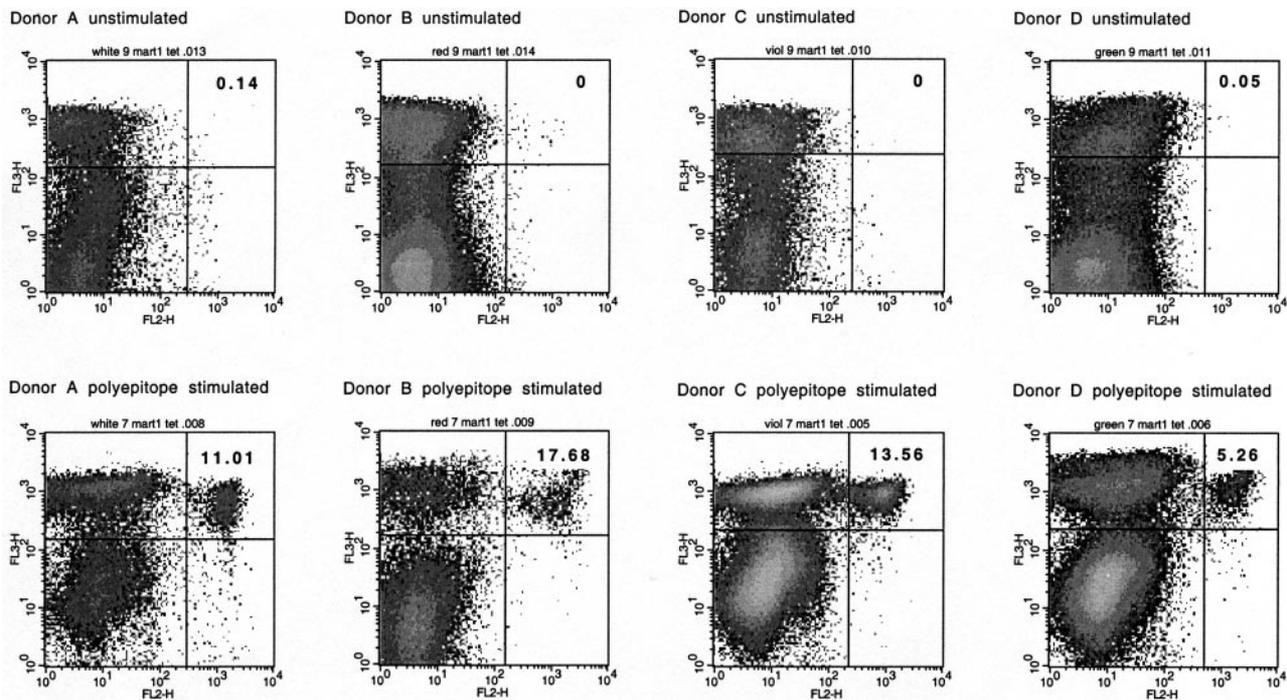


Fig. 3 Staining of unstimulated and poly-MEL stimulated lymphocytes with MART-1-specific tetrameric HLA-A2. Freshly derived lymphocytes (*ex vivo*) and CTL taken from cultures primed and boosted with DC/poly-MEL were stained for MART-1-reactive T-cell receptor using fluorogenic HLA-A2-tetramers and counterstained with anti-CD8. The results are from four donors (A–D), and the percentage of tetramer-positive CD8+ T cells is shown in each plot.

ity of epitopes encoded but that its repeated use can lead to the generation of an immunodominant response.

Polyepitope protein was shown to be translated in agreement with others (12). However, despite generating immune responses some other groups have not detected polyepitope proteins (8, 14). In our study although polyepitope could be found in COS7 cells, we did not see it in virally transduced DC (data not shown). We have observed large variations previously in the abundance of different polyepitope proteins in transfected cells.⁴ Variation in polyepitope abundance may reflect differences in mRNA stability or be a consequence of protein stability. This may relate to the ability of different polyepitope proteins to form secondary and tertiary structures and, hence, resist proteolytic processes within the cell (8).

Our study demonstrates priming of CTL responses specific for up to 6 of 6 HLA-A2 epitopes by transduced human DC. To our knowledge, this is the first report of multiple CTL responses induced by a polyepitope in a human system. Heemskerk *et al.* (18) used human CD34+ -derived DC transduced with retrovirally vectored polyepitope and demonstrated a single CTL response to the influenza matrix_(58–66) epitope. In an HLA-A2 transgenic murine model, 4 of 7 and 7 of 10 A2-restricted epitopes induced CTL responses after vaccination with HIV and melanoma polyepitopes, respectively (14, 16). These experiments involved polyepitope

priming and boosting *in vivo* followed by splenocyte stimulation *in vitro* with peptide-sensitized blast cells, thus paralleling the *in vitro* experiments in our study. Although these experiments used DNA constructs encoding several antigens that are incorporated in poly-MEL, several differences were seen. The polyepitope used by Mateo *et al.* (16) failed to induce any responses against the tyrosinase_{1–9} or the MAGE-3_{271–279} epitopes. In contrast, in human cells responses were seen in 1 of 4 and 3 of 4 donors in our study. This may relate to the inability of HLA-A2 transgenic mice to respond to some epitopes because of a limited T-cell repertoire or to differences in TAP processing. Indeed, vaccination of HHD mice with either tyrosinase_{1–9} or the MAGE-3_{271–279} peptides also failed to elicit CTL (16). Alternatively this may be in part attributable to differences in the two polyepitope constructs. Thus, though sophisticated transgenic mice provide invaluable preclinical models for testing such therapeutic approaches, studies in a fully human system can give key preclinical data not available from murine models.

Multiple stimulation with poly-MEL-transduced DC led to immunodominance with generation of CTLs to only one epitope. The reasons for this are unclear but as suggested by Yewdell and Bennink (30), may include binding of peptide to HLA, effect of precursor frequency, more efficient liberation of dominant epitopes by DCs, and suppression of T-cell reactive to subdominant epitopes by those specific for immunodominant epitopes. The affinity of epitope for HLA is unlikely to simply account for the immunodominance, because the predicted affin-

⁴ Unpublished observations.

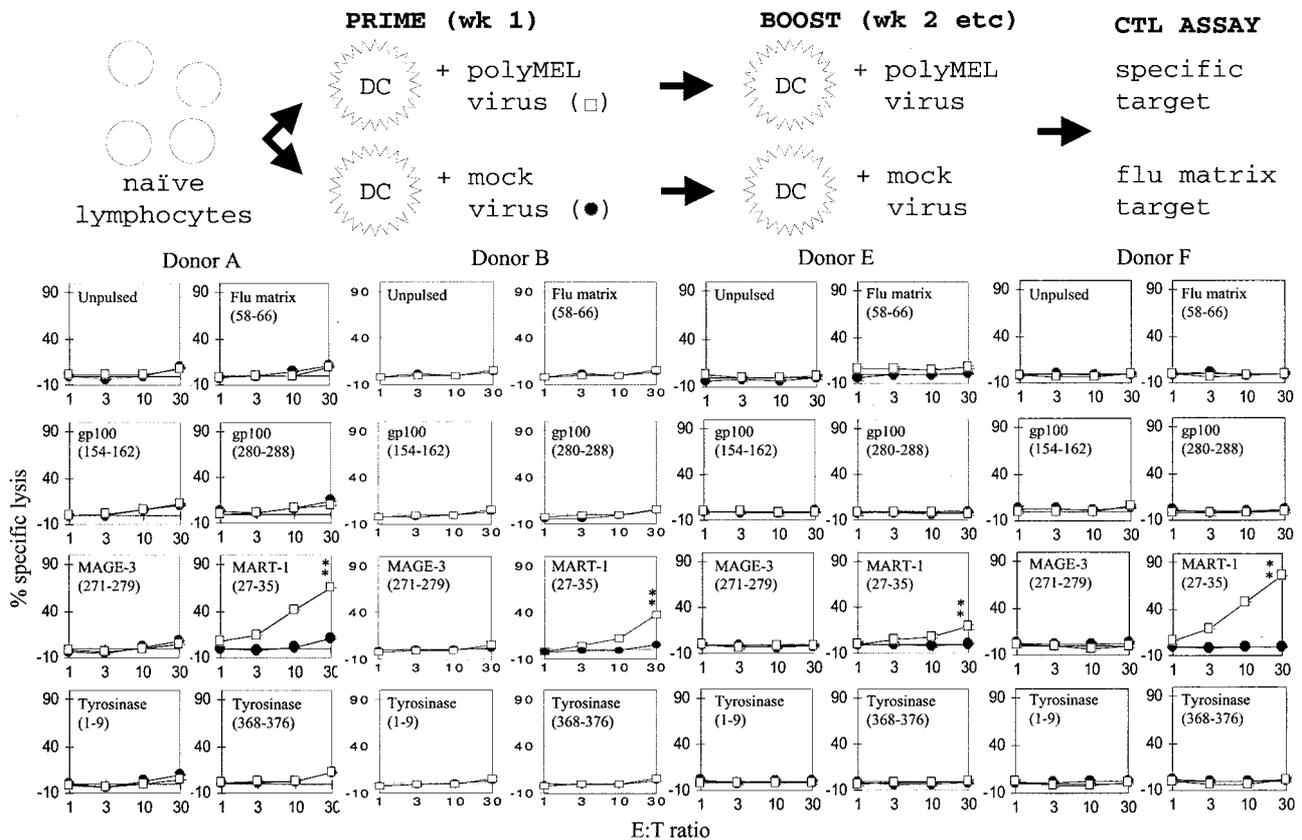


Fig. 4 Repeated stimulation with poly-MEL leads to immunodominance. CTL responses after repeated stimulation of autologous PBL with transduced MD-DC, shown in cartoon form. Responder PBL received up to 5 weekly stimulations with irradiated, poly-MEL- (□) or mock- (●) transduced MD-DC. Responder lymphocytes were tested for specific cytotoxicity against ^{51}Cr -labeled T2 cells pulsed with the indicated peptide epitope. Each point represents the mean and SD of triplicate experiments. Significant differences in cytotoxicity mediated by polyepitope and control induced effectors at the 30:1 E:T ratio are indicated (** $P < 0.001$). Representative results from four donors are given.

ity of the MART epitope for HLA-A2 is lower than most other HLA-A2-binding epitopes in poly-MEL. Although a full study of precursor frequency of specific CTLs was not undertaken with our donors, the *ex vivo* PBL of two of four donors had a demonstrable precursor population of MART-1-reactive CD8⁺ T cells. Furthermore, a large proportion of HLA-A2⁺ healthy donors (60%) are known to possess high frequencies of MART-1-specific CTLs (31). Our own data⁵ confirmed this observation, showing that 3 of 10 normal HLA-A2⁺ donors have a significant proportion of MART-1-specific CD8⁺ cells as judged by reactivity with MART-1 tetramer. By contrast, no precursors to the other antigens in our polyepitope were detected by specific tetramers. Polyepitope responses have been investigated in the presence of large numbers of preexisting CTL populations (32) and indicated that strong, existing responses inhibited vaccine-induced responses that were restricted by the same MHC haplotype. This is in agreement with the observation that a high-frequency precursor number is a contributory mechanism for immunodominance of some epitopes (33). Importantly,

the abundance of professional antigen presenting cells may contribute to immunodominance as T cells compete for antigen displayed by adenomatous polyposis coli (34). Kedl *et al.* (34) demonstrated competition between high-affinity OVA-specific transgenic OT1 T cells and either subdominant OVA epitopes or unrelated epitopes. In this instance, introduction of large numbers of additional DC overcame the immunodominance by OT1 cells. It remains to be determined whether the abundance of DCs in our model *in vitro* system was responsible for the observed immunodominance.

There is a possibility that the quantity of epitope presented by polyepitope-expressing DCs is directly related to the MART-1 immunodominance observed. A previous study described an immunodominant HLA-A11-restricted CTL epitope from the EBNA3B protein of EBV that is more abundant than a related, subdominant epitope (35). Although the MART-1 epitope is predicted to bind with a much lower affinity to HLA-A2 than all but one of the A2-restricted epitopes in poly-MEL, it may be liberated from the full length polyepitope protein much more efficiently and, thus, achieve a greater availability for loading of HLA molecules than the other epitopes. Although quantitation of epitope abundance could be achieved after elution of epitopes from the MHC molecules of poly-

⁵ Unpublished observations.

epitope-expressing cells, it should be noted that immunodominance does not necessarily correlate with epitope abundance on the cell surface. It was recently demonstrated by Crotzer *et al.* (36) that the immunodominant EBV-derived epitope RRIYD-LIEL is in fact the least abundant of four EBV-derived epitopes presented by HLA-B27.

In conclusion, we have described a polyepitope vaccine designed to induce CTL responses to commonly expressed melanoma antigens. We demonstrated that the majority of HLA-A2 restricted epitopes are processed and presented by human DC and effectively prime CTL responses *in vitro*. However, as the response was restricted to a single epitope after repeated polyepitope stimulation, vaccination strategies may need to consider the relative immunodominance of epitopes included in polyepitopes and vaccination schedules. These questions are to be addressed in a DNA vaccination clinical trial in patients with malignant melanoma.

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