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HLA-Independent Heterogeneity of CD8^{+} T Cell Responses to MAGE-3, Melan-A/MART-1, gp100, Tyrosinase, MC1R, and TRP-2 in Vaccine-Treated Melanoma Patients[1]

Sandra R. Reynolds,* Esteban Celis,[2†] Alessandro Sette,[†] Ruth Oratz,[‡] Richard L. Shapiro,[§] Dean Johnston,* Marilena Fotino[¶], and Jean-Claude Bystryn[3]*

An important element in melanoma vaccine construction is to identify peptides from melanoma-associated Ags that have immunogenic potential in humans and are recognized by CD8^{+} T cells in vivo. To identify such peptides, we evaluated HLA-A*02^{+} melanoma patients immunized to a polyvalent vaccine containing multiple Ags, including MAGE-3, Melan-A/MART-1, gp100, tyrosinase, melanocortin receptor (MC1R), and dopachrome tautomerase (TRP-2). Using a filter spot assay, we measured peripheral blood CD8^{+} T cell responses, before and after immunization, to a panel of 45 HLA-A*0201-restricted peptides derived from these Ags. The peptides were selected for immunogenic potential based on their strong binding affinity in vitro to HLA-A*0201. Vaccine treatment induced peptide-specific CD8^{+} T cell responses to 22 (47.8%) of the peptides. The most striking finding was the HLA-independent heterogeneity of responses to both peptides and Ags. All responding patients reacted to different combination of peptides and Ags even though the responding patients were all A*0201^{+} and the peptides were all A*0201-restricted. From 9 to 27% of patients developed a CD8^{+} T cell response to at least one peptide from each Ag, but no more than 3 (14%) reacted to the same peptide from the same Ag. This heterogeneity of responses to individual peptides and Ags in patients with the same haplotype points to the need to construct vaccines of multiple peptides or Ags to maximize the proportion of responding patients. ***The Journal of Immunology,*** **1998, 161: 6970–6976.**

An important requirement for vaccines against cancer is that they stimulate antitumor CD8^{+} T cells, as these are thought to be the major effector cells in tumor rejection (1, 2). There is an interest in finding peptides that have the ability to stimulate such responses because these peptides would be good candidates for vaccine construction. A number of tumor-derived, HLA-restricted peptides have been identified that are antigenic, i.e., recognized by CD8^{+} T cells in vitro (3–13). However, their immunogenicity remains unknown, as this requires direct evidence that they can stimulate CD8^{+} T cell responses in vivo when used to immunize patients. Recently, we developed a sensitive method to quantify peptide-specific CD8^{+} T cells in peripheral blood so that the small increase in the number of these cells induced by active immunization to Ag can be detected. Using this procedure (14), we directly detected without in vitro sensitization, vaccine-induced CD8^{+} T cell responses to two peptides derived from the melanoma-associated Ags MAGE-3$_{271–278}$ (3) and Melan-A/MART-1$_{27–35}$ (4).

We have now extended these studies by examining patients' responses in vivo to a large number of peptides derived from Ags expressed by human melanoma cells. These include MAGE-3 (15), Melan-A/MART-1 (16), gp100 (5), tyrosinase, melanocortin receptor (MC1R)[4] (17), and dopachrome tautomerase (TRP-2) (18, 19), all of which are known to be antigenic in vitro. The study focused on HLA-A*0201-restricted peptides, the allele most commonly expressed by patients at risk for developing melanoma.

Materials and Methods

Patients

We evaluated 22 sequential, vaccine-treated, HLA-A*02^{+} patients with resected malignant melanoma. Seven (32%) had American Joint Committee on Cancer (AJCC) stage II, nine (41%) had stage III and six (27%) had stage IV disease. HLA typing was performed by complement-mediated cytotoxicity. HLA-A2 subtyping was performed by the PCR-SSP (sequence specific primers) technique using the set containing 5′ and 3′ primers for identifying A*0201 to A*0217 alleles (Dynal, Oslo, Norway) (20).

Vaccine and immunization

All patients were immunized to a polyvalent melanoma vaccine prepared, as previously described, from Ags shed into culture medium by a pool of melanoma cell lines (SFM14, SFM20 and SFSKMel28) (21, 22). Briefly, the shed material was collected, concentrated, pooled, treated with 0.5% Nonidet P-40 to break up aggregates, and ultracentrifuged at 100,000 × *g* for 90 min to deplete alloantigens. The supernatant was filter sterilized, adjusted to a protein concentration of 200 μg/ml, dispensed into sterile glass vials, and frozen at −80°C until used. The vaccine contains MAGE-1, MAGE-3, Melan-A/MART-1, and tyrosinase by Western blotting (14), gp-100 by immune precipitation, and several other melanoma-associated Ags of 45–110 kDa that are expressed by melanoma tissue in vivo and are immunogenic in humans (23). All patients were immunized to vaccine, using alum or liposomes as adjuvant (24). It was administered intradermally split into the four extremities, every 2–3 wk four times. Peripheral blood was collected before immunization and 1 wk following the fourth immunization. Mononuclear cells were separated on Ficoll-hypaque and frozen in liquid N_2 until used.

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[4] Abbreviations used in this paper: MC1R, melanocortin receptor; TRP-2, dopachrome tautomerase; AJCC, American Joint Committee on Cancer.

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Preparation of peptides

Peptides were prepared on an Applied Biosystems (Foster City, CA) instrument. Briefly after removal of the α-amino-*ter*-butylcarbonyl protecting group, the phenylacetamidomethyl resin peptide was coupled with a 4-fold excess of preformed symmetrical anhydride (hydroxybenzyltriazole esters for arginine, histidine, asparagine, and glutamine) for 1 h in dimethylformamide. For arginine, histidine, asparagine, glutamine, and histidine residues, the coupling step was repeated to obtain a high efficiency coupling. Peptides were cleaved by treatment with hydrogen fluoride in the presence of the appropriate scavengers. Synthetic peptides were purified by reverse phase HPLC. The purity and identity of the peptides, which was routinely >95% was determined by amino acid sequence and mass spectrometric analysis respectively.

Assay of peptide binding affinity to HLA-A*0201

Peptide binding to purified HLA-A*0201 molecules was measured as previously described (25). Briefly, the assay is based on the inhibition of binding to detergent-solubilized HLA molecules of a radiolabeled standard peptide with strong binding affinity for HLA-A*0201. The standard peptide, FLPSDYFPSV, was radioiodinated by the chloramine T method using ^{125}I (ICN, Irvine, CA). HLA-A*0201 concentration yielding ~15% of bound peptide (approximately in the 10 nM range) was used in the inhibition assays. Various doses of the test peptides (1 nM to 10 μM) were incubated with 5 nM radiolabeled standard peptide and HLA-A*0201 molecules for 2 days at room temperature in the presence of a mixture of protease inhibitors and 1 μM β_2-microglobulin (Scripps Laboratories, San Diego, CA). At the end of the incubation period, the percent HLA bound radioactivity was determined by gel filtration. The peptides were selected based on their capacity to bind to HLA-A*0201 with an affinity greater than an IC_{50} of 500 nM, which is known to be in the immunogenic range for cytotoxic T lymphocyte epitopes (26).

Assay of peptide-specific CD8$^+$ T cells

We determined the number of peptide-specific CD8$^+$ T cells in peripheral blood by filter spot assay as previously described (14). Briefly, 96-well polyvinylidene difluoride filter plates (Millipore, Bedford, MA) were precoated with mAbs to human IFN-γ, (Biosource, Camarillo, CA) and seeded with ~20,000 HLA-A*0201$^+$ target cells/well (SFM20 · A2) (14). The target cells were pulsed with 20 nM of test peptide before addition of effectors. The HLA-A*0201-restricted influenza peptide FluM1$_{58-66}$ (GILGFVFTL) to which most individuals have a CD8$^+$ T cell response was added to some wells as a positive control (27). mAb IVA12 (anti-HLA-DR, DP, and DQ (HB145 from the American Type Culture Collection, Manassas, VA) was added to all wells to prevent presentation of class II Ags by targets or residual monocytes. Effector PBMC were thawed, resuspended in RPMI 1640 with 10% FBS and depleted of monocytes on plastic. The cells were then added to the wells, incubated 48 h at 37°C and 5% CO_2, washed with PBS-Tween 20, incubated overnight with goat anti-IFN-γ (R&D, Minneapolis, MN), followed by biotinylated rabbit anti-goat Ig (Sigma, St. Louis, MO) and extravidin alkaline phosphatase (Sigma). Spots representing individual T cells that had been stimulated by peptides and released IFN-γ were visualized with BCIP/NBT (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and counted with a dissecting microscope.

The number of CD8$^+$ T cells was calculated as the number of IFN-γ-secreting cells in wells without Abs minus those in wells with anti-CD8 Abs (OKT8 from the American Type Culture Collection). The number of peptide-specific CD8$^+$ T cells was determined by subtracting the number of CD8$^+$ cells reacting to targets pulsed with an A*0201-restricted control peptide (YLKKIKNSL from falciparum malaria) (28) from the number of CD8$^+$ T cells reacting to melanoma peptide-pulsed targets. All assays were conducted twice, once in duplicate and once in triplicate, and the average value used. The mean SD was ±0.95. The test was considered positive if there were ≥5 peptide-specific CD8$^+$ T cells per 500,000 PBL, which is twice the maximum SD. The number of vaccine-induced peptide-specific CD8$^+$ T cells was calculated by subtracting the number of peptide-specific CD8$^+$ T cells present before treatment from that after four vaccine immunizations in the same patient. The vaccine was considered to have stimulated a CD8$^+$ T cell response to that peptide if this number was ≥5.

Results

Selection of peptides

The peptides used in this study were selected for their potential ability to stimulate CD8$^+$ T cells in vivo based on being derived from Ags known to be associated with melanoma, i.e., MAGE-3, Melan-A/MART-1, gp100, tyrosinase, MC1R, or TRP-2; having 9–10 amino acid residues; containing an HLA-A*0201 binding motif; and by having high binding affinity for HLA A*0201 in vitro. A total of 45 melanoma peptides that satisfied these criteria is provided in Table I.

CD8$^+$ T cell responses to individual peptides

We examined the ability of the 45 HLA-A*0201-restricted peptides selected as described above, an HLA-A*0201-restricted malaria peptide as a negative control (28), and an HLA-A*0201-restricted influenza peptide as a positive control (27) to be recognized by CD8$^+$ T cells from melanoma patients. We measured the number of CD8$^+$ T cells directed to each peptide before and after immunization to a polyvalent melanoma vaccine containing the Ags from which the melanoma peptides were derived. The study was conducted on 22 sequential HLA-A*02$^+$ patients who were immunized to the polyvalent vaccine. All but one patient were A*0201$^+$. Patient 484 was A*0205+.

Before vaccine treatment, elevated numbers of circulating peptide-specific CD8$^+$ T cells were found in 8 of the 22 patients. These patients reacted to 14 of the 45 melanoma peptides (see Table I). One or more of these peptides was derived from each of the 7 Ags studied. However, elevated CD8$^+$ T cells to any one peptide were present in no more than 1 patient (5%).

Following four vaccine immunizations, a peptide-specific CD8$^+$ T cell response was induced or augmented to 22 (47.8%) of the peptides, as indicated by a minimum increase of 5 peptide-specific CD8$^+$ T cells over baseline measurement in the same patient (see Table II). Of the induced responses, at least one of the inducing peptides was derived from each of the proteins studied. The most frequently positive peptides were gp100$_{585-613}$ IMPGQEAGL) to which responses were induced in 3 (14%) of the 22 patients (see Table II). The highest vaccine-induced responses were directed to MAGE-3$_{271-278}$ (FLWGPRALV), Melan-A/MART-1$_{56-64}$ (ALMDKSLHV) and gp100$_{13-21}$ (AVIGALLAV) which all were >40 peptide-specific CD8$^+$ T cells/500,000 PBL over baseline measurements. Twenty-one of the 22 patients were also tested for their ability to recognize the HLA-A*0201-restricted influenza peptide FluMI$_{58-66}$ (27) as a positive control. This peptide was recognized by 76% of the patients. There was no response to the control malaria peptide in any patient either before or after vaccine immunization. Overall, vaccine immunization induced a peptide specific CD8$^+$ T cell response to at least one peptide in 59% of the 22 patients. Responses were induced in 7 of 14 (50%) of patients without a pre-existing response, and further induced in 4 of 8 (50%) patients who had a pre-existing response before vaccine treatment.

Relative responses to individual Ags

The relative responses to each Ag were evaluated based on the frequency of vaccine-induced, peptide-specific, CD8$^+$ T cell responses to at least one peptide derived from that Ag. The results are summarized in Table III. All of the melanoma Ags stimulated a CD8$^+$ T cell response to at least one peptide on that Ag. The most frequently recognized Ag was gp100, which induced CD8$^+$ T cell responses in 27% of patients. Overall, 59% of the patients

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Table I. *Pre-immunization responses to HLA-A2-restricted peptides*

Protein	Peptide	IC_{50} (nM)[a]	Pre-Existing CD8+ T Cell Response: % Patients with recognition[b] (n = 22)	Pre-Existing CD8+ T Cell Response: Response level[c]
MAGE-3$_{271-279}$	FLWGPRALV	61.0	5	18.7
MAG-3$_{188-198}$	IMPKAGLLIIV	20.0	0	
MAGE-3$_{159-169}$	QLVFGIELMEV	7.9	0	
Melan-A/MART-1$_{31-39}$	GILTVILGV	4.6	5	8.3
Melan-A/MART-1$_{56-64}$	ALMDKSLHV	17.2	5	6.6
Melan-A/MART-1$_{27-35}$	AAGIGILTV	1063.8	5	8.5
gp100$_{178-186}$	MLGTHTMEV	14.3	0	
gp100$_{13-21}$	AVIGALLAV	17.9	5	21.4
gp100$_{250-259}$	YLAEADLSYT	45.5	0	
gp100$_{619-627}$	RLMKQDFSV	46.3	0	
gp100$_{177-186}$	AMLGTHTMEV	51.9	5	5.5
gp100$_{585-593}$	IMPGQEAGL	64.9	0	
gp100$_{11-19}$	HLAVIGALL	106.4	0	
gp100$_{606-614}$	LMAVVLASL	111.1	0	
gp100$_{232-241}$	FLRNQPLTFA	138.9	5	10.3
gp100$_{570-578}$	SLADTNSLA	454.6	0	
Tyrosinase$_{9-17}$	LLWSFQTSA	15.2	0	
Tyrosinase$_{8-17}$	BLLWSFQTSA	19.2	5	6.3
Tyrosinase$_{214-222}$	FLLRWEQEI	25.6	0	
Tyrosinase$_{477-486}$	WLLGAAMVGA	32.1	0	
Tyrosinase$_{2-10}$	LLAVLYCLL	33.3	0	
Tyrosinase$_{490-499}$	ALLAGLVSLL	56.4	0	
Tyrosinase$_{482-490}$	AMVGAVLTA	75.5	0	
Tyrosinase$_{490-498}$	ALLAGLVSL	76.3	5	10.3
Tyrosinase$_{487-495}$	VLTALLAGL	82.0	5	15.6
Tyrosinase$_{491-499}$	LLAGLVSLL	95.2	0	
Tyrosinase$_{473-481}$	RIWSWLLGA	100.0	0	
Tyrosinase$_{207-215}$	FLPWHRLFL	112.3	0	
Tyrosinase$_{207-216}$	FLPWHRLFLL	204.9	0	
MC1R$_{99-197}$	LLLEAGALV	6.7	0	
MC1R$_{251-259}$	FLCWGPFFL	38.5	5	12.5
MC1R$_{291-299}$[d]	SVMDPLIYA	83.3	0	
MC1R$_{9-17}$	RLLGSLNST	166.7	0	
MC1R$_{291-299}$	AIIDPLIYA	238.1	0	
MC1R$_{79-87}$	CLALSDLLV	357.1	0	
MC1R$_{291-299}$[d]	SIIDPLIYA	454.6	0	
TRP-2$_{431-439}$	NMVPFFPPV	3.9	0	
TRP-2$_{185-193}$	FVWLHYYSV	15.2	0	
TRP-2$_{455-463}$	YAIDLPVSV	15.6	5	6.7
TRP-2$_{288-296}$	SLDDYNHLV	26.3	0	
TRP-2$_{476-484}$	VMGTLVALV	27.8	0	
TRP-2$_{180-188}$	SVYDFFVWL	35.7	5	6.4
TRP-2$_{475-483}$	VVMGTLVAL	66.7	0	
TRP-2$_{156-164}$	YVITTQHWL	104.2	0	
TRP-2$_{367-376}$	SLHNLVHSFL	263.2	5	19.0
CSP$_{334-342}$	YLKKIKNSL	NT[e]	0	

[a] IC_{50} is defined as the concentration of peptide required to inhibit 50% of the binding of a test peptide to purified HLA-A*0201 molecules.

[b] The percent of patients out of those tested, who recognized the peptide.

[c] The actual CD8+ T cell responses, i.e. the number of IFN-γ-secreting DC8+ T cells/500,000 PBL. These were calculated by subtracting the number of IFN-γ-secreting cells in wells treated with anti-CD8 from wells without Abs, both in the presence of the same peptide. Mean standard deviation was ±0.95.

[d] Analog (three amino acids have been changed from the original sequence).

[e] NT, not tested.

had a vaccine-induced CD8+ T cell response to at least one of the Ags studied.

Heterogeneity of CD8+ T cell responses to individual Ags and peptides

There was striking heterogeneity in the ability of individual patients to develop vaccine-induced CD8+ T cell responses to individual peptides on the same Ag. An example of this heterogeneity in responses to peptides of gp100 is illustrated in Table IV. Individual patients immunized to the same amount of gp100-containing vaccine developed CD8+ T cell responses to one gp100 peptide but not to others, whereas the reverse was true of other patients. Overall, 27% patients developed a CD8+ T cell response to at least one of the gp100 peptides, but no

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Table II. *Post-immunization responses to HLA-A2-restricted peptides*

Protein	Peptide	IC_{50} (nM)[a]	Vaccine-Induced $CD8^+$ T Cell Response: % Patients with response[b] (n = 22)	Vaccine-Induced $CD8^+$ T Cell Response: Response level[c]
MAGE-$3_{271-279}$	FLWGPRALV	61.0	14	10.6, 89.2, −17.6[d,e]
MAGE-$3_{188-198}$	IMPKAGLLIIV	20.0	5	8.2
MAGE-$3_{159-169}$	QLVFGIELMEV	7.9	9	11.8, 5.8
Melan-A/MART-1_{31-39}	GILTVILGV	4.6	5	8.3
Melan-A/MART-1_{56-64}	ALMDKSLHV	17.2	9	45.5,[f] 12.7
Melan-A/MART-1_{27-35}	AAGIGILTV	1063.8	5	11.7[f]
$gp100_{178-186}$	MLGTHTMEV	14.3	5	13.0
$gp100_{13-21}$	AVIGALLAV	17.9	5	69.5[f]
$gp100_{250-259}$	YLAEADLSYT	45.5	0	
$gp100_{619-627}$	RLMKQDFSV	46.3	0	
$gp100_{177-186}$	AMLGTHTMEV	51.9	5	−5.5[e]
$gp100_{585-593}$	IMPGQEAGL	64.9	14	6.1, 8.4, 10.7
$gp100_{11-19}$	HLAVIGALL	106.4	5	7.7
$gp100_{606-614}$	LMAVVLASL	111.1	0	
$gp100_{232-241}$	FLRNQPLTFA	138.9	5	−10.3[e]
$gp100_{570-578}$	SLADTNSLA	454.6	5	14.8
$Tyrosinase_{9-17}$	LLWSFQTSA	15.2	0	
$Tyrosinase_{8-17}$	BLLWSFQTSA	19.2	9	11.5, −6.3[e]
$Tyrosinase_{214-222}$	FLLRWEQEI	25.6	5	22.4
$Tyrosinase_{477-486}$	WLLGAAMVGA	32.1	0	
$Tyrosinase_{2-10}$	LLAVLYCLL	33.3	0	
$Tyrosinase_{490-499}$	ALLAGLVSLL	56.4	0	
$Tyrosinase_{482-490}$	AMVGAVLTA	75.5	9	6.8, 17.1
$Tyrosinase_{490-498}$	ALLAGLVSL	76.3	5	21.8
$Tyrosinase_{487-495}$	VLTALLAGL	82.0	9	9.3, 13.4
$Tyrosinase_{491-499}$	LLAGLVSLL	95.2	0	
$Tyrosinase_{473-481}$	RIWSWLLGA	100.0	0	
$Tyrosinase_{207-215}$	FLPWHRLFL	112.3	0	
$Tyrosinase_{207-216}$	FLPWHRLFLL	204.9	0	
$MC1R_{99-197}$	LLLEAGALV	6.7	0	
$MC1R_{251-259}$	FLCWGPFFL	38.5	9	23.4[f]
$MC1R_{291-299}$[g]	SVMDPLIYA	83.3	0	
$MC1R_{9-17}$	RLLGSLNST	166.7	0	
$MC1R_{291-299}$	AIIDPLIYA	238.1	5	22.2
$MC1R_{79-87}$	CLALSDLLV	357.1	5	13.7
$MC1R_{291-299}$[g]	SIIDPLIYA	454.6	0	
TRP-$2_{431-439}$	NMVPFFPPV	3.9	0	
TRP-$2_{185-193}$	FVWLHYYSV	15.2	0	
TRP-$2_{455-463}$	YAIDLPVSV	15.6	9	37.1[f], 36.1
TRP-$2_{288-296}$	SLDDYNHLV	26.3	0	
TRP-$2_{476-484}$	VMGTLVALV	27.8	0	
TRP-$2_{180-188}$	SVYDFFVWL	35.7	9	16.6, −6.4[e]
TRP-$2_{475-483}$	VVMGTLVAL	66.7	0	
TRP-$2_{156-164}$	YVITTQHWL	104.2	0	
TRP-$2_{367-376}$	SLHNLVHSFL	263.2	9	15.4, −19.8[e]
$CSP_{334-342}$	YLKKIKNSL	NT[h]	0	

[a] IC_{50} is defined as the concentration of peptide required to inhibit 50% of the binding of a test peptide to purified HLA-A*0201 molecules.
[b] The percent of patients out of those tested, who recognized the peptide.
[c] The actual $CD8^+$ T cell responses, i.e. the number of IFN-γ-secreting $CD8^+$ T cells/500,000 PBL. These were calculated by subtracting the number of IFN-γ-secreting cells in wells treated with anti-CD8 from wells without Abs, both in the presence of the same peptide. Mean standard deviation was ±0.95.
[d] More than one value indicates multiple patient responses.
[e] −, Response decreased after vaccine immunization.
[f] Response increased further by vaccine immunization.
[g] Analog (three amino acids have been changed from the original sequence).
[h] NT, Not tested.

more than three patients (14%) reacted to the same peptide. There was a similar degree of heterogeneity in vaccine-induced responses to peptides derived from the other Ags (data not shown). Nine to 18% of the patients reacted to at least one peptide from each Ag, but no more than three patients (14%) reacted to the same peptide that Ag.

There was also marked heterogeneity in the responses of individual patients to individual Ags. As illustrated in Table III, some

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Table III. *Vaccine-induced CD8+ T cell responses to melanoma-associated Ags*

Patient ID	gp100	Tyrosinase	Melan-A/MART-1	MAGE-3	TRP-2	MC1R	Any Peptide
191	6.1[a]	0[b]	0	0	0	0	+
375	0	0	45.5	89.2	0	0	+
418	0	0	8.3	10.6	0	0	+
422	0	0	0	11.8	0	0	+
436	10.7, −10.3[c]	22.4, −6.3	0	0	36.1, 16.6[d]	23.4, 13.7	+
442	0	0	0	8.2, 5.8	0	0	+
443	−5.5	0	0	0	0	0	−
445	0	0	0	0	0	0	−
458	7.7	0	11.7	0	0	22.2	+
460	13.0	11.5	0	0	0	0	+
462	0	0	0	0	0	0	−
465	0	0	0	0	0	0	−
467	0	21.8, 6.8	0	0	0	0	+
468	69.5, 8.4	0	0	0	37.1, −19.0	0	+
471	0	0	0	0	0	0	−
475	0	0	0	−17.6	0	0	−
477	0	0	0	0	0	0	−
478	0	17.1	12.7	0	−6.4	0	+
479	0	13.4	0	0	15.4	0	+
482	14.8	0	0	0	0	0	+
483	0	0	0	0	0	0	−
484	0	0	0	0	0	0	−
%	27%	23%	18%	18%	13%	9%	59%

[a] Number of vaccine-induced peptide-specific CD8+ T cells/500,000 PBL. These were calculated by subtracting the number of peptide-specific CD8+ T cells present prior to immunization from that after four immunizations in the same patient.
[b] 0, Values that were between −4.9 and 4.9 after subtraction of the preimmunization response. This range on either side of 0 is approximately twice the maximum standard deviation of our assay.
[c] A value with a − indicates that the pre-immunization response was greater than the postimmunization response.
[d] Multiple values indicate stimulation of CD8+ T cells to more than one peptide on the same Ag.

patients developed vaccine-induced CD8+ T cell responses to peptides of one Ag but not to those of another Ag, whereas the reverse was true for other patients. Though 59% of patients developed CD8+ T cell responses to at least one of seven Ags studied, only 9% to 27% of patients reacted to the same Ag.

Correlation of vaccine-induced CD8+ T cell responses with clinical outcome

The clinical status of the patients in the study is shown in Table V. All survived beyond 1 year, and over 75% are still surviving. Of those patients who had a vaccine-induced CD8+ T cell response to at least one of the Ags tested, a higher proportion (though not statistically significant) were progression-free after 1 year as compared with those who had no response (54% vs 22%). This positive correlation was unrelated to the stage of disease or the type of adjuvant administered with the vaccine as these two variables were equally distributed in both groups.

Table IV. *Heterogeneity in CD8+ T cell responses to gp100 peptides*

gp100 Peptide	Patient ID: 191	436	458	460	468	482
MLGTHTMEV	0	0	0	13.0[a]	0[b]	0
AVIGALLAV	0	0	0	0	69.5	0
YLAEADLSYT	0	0	0	0	0	0
RLMKQDFSV	0	0	0	0	0	0
AMLGTHTMEV	0	0	0	0	0	0
IMPGQEAGL	6.1	10.7	0	0	8.4	0
HLAVIGALL	0	0	7.7	0	0	0
LMAVVLASL	0	0	0	0	0	0
FLRNQPLTFA	0	−10.3	0	0	0	0
QLVFGIELMEV	0	0	0	0	0	14.8

[a] Number of vaccine-induced peptide-specific CD8+ IFN-γ-secreting T cells/500,000 PBL. These were calculated by subtracting the number of peptide-specific CD8+ T cells present prior to immunization from that after four immunizations in the same patient.
[b] 0, Values that were between −4.9 and 4.9 after subtraction of the pre-immunization response. This range on either side of 0 is approximately twice the maximum standard deviation of our assay.

Table V. *Clinical data*

Patient ID	HLA Type	Stage[a]	Adjuvant	Vaccine-Induced CD8+ T Cell Response[b]	Progression-Free at 1 Year
445	A*0201	II	IL-2 lip[c]	−	Yes
475	A*0201	IV	Alum[d]	−	Yes
443	A*0201	III	Alum	−	No
471	A*0201	IV	IL-2 lip	−	No
462	A*0201	III	IL-2 lip	−	No
477	A*0201	II	IL-2 lip	−	No
483	A*0201	III	IL-2 lip	−	No
484	A*0205	II	IL-2 lip	−	No
465	A*0201	IV	Alum	−	No
436	A*0201	III	IL-2 lip	+	Yes
191	A*0201	III	IL-2 lip	+	Yes
375	A*0201	III	IL-2 lip	+	Yes
422	A*0201	III	IL-2 lip	+	Yes
442	A*0201	IV	IL-2 lip	+	Yes
460	A*0201	II	IL-2 lip	+	Yes
468	A*0201	II	IL-2 lip	+	Yes
458	A*0201	III	Alum	+	No
418	A*0201	IV	IL-2 lip	+	No
482	A*0201	IV	IL-2 lip	+	No
467	A*0201	III	IL-2 lip	+	No
478	A*0201	II	IL-2 lip	+	No
479	A*0201	II	IL-2 lip	+	No

[a] AJCC stage diagnosis at time of first vaccine immunization.
[b] Vaccine-induced CD8+ T cell response to one or more of the studied Ags.
[c] Vaccine was encapsulated in liposomes along with IL-2.
[d] Vaccine was bound to alum.

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Most likely a larger study will be needed to show statistically significant differences.

Discussion

The major findings in this study are the identification of multiple melanoma-associated peptides that can stimulate $CD8^+$ T cell responses in vivo in humans, and the demonstration of marked HLA-independent heterogeneity in the ability of these peptides to induce $CD8^+$ T cell responses in different individuals. A multipronged strategy was used to identify these peptides. 1) We immunized patients to a polyvalent vaccine that contains multiple melanoma-associated Ags. This permitted the direct and concurrent identification of multiple immunogenic peptides by measuring the presence and level of peptide-specific $CD8^+$ T cells in peripheral blood before and after vaccine treatment. The vaccine contains MAGE-3, Melan-A/MART-1, gp100, and tyrosinase (14). It also contains MC1R and TRP-2, as shown in this study by its ability to stimulate $CD8^+$ T cell responses to these Ags. 2) We used a screening strategy to select peptides that had a high potential to stimulate $CD8^+$ T cells. It was based on their having 9–10 amino acid residues, an anchor motif, and high binding affinity to, HLA-A*0201 in vitro. This made it feasible to focus studies on those peptides most likely to be immunogenic. 3) We used a sensitive filter spot assay for peptide-specific $CD8^+$ T cells to evaluate the response to these peptides in vivo. The assay could detect fewer than 5 peptide-specific $CD8^+$ T cells per 500,000 PBL (14) and was sensitive enough to quantify the small increase in peptide-specific $CD8^+$ T cells which appears in peripheral blood following vaccine immunization. Our method was reproducible within ± 2.0 IFN-γ-producing cells/500,000 PBL and required only a small amount of the blood with minimum manipulation compared with assays using purified $CD8^+$ T cells. Using this strategy, we identified multiple peptides presented by HLA-A*0201 and derived from MAGE-3, Melan-A/MART-1, gp100, tyrosinase, MC1R, and TRP-2 that have immunogenic potential as evidenced by their ability to stimulate $CD8^+$ T cell responses in vivo. Several of these peptides were previously known to be recognized by $CD8^+$ T cells in vitro, including FLWGPRALV from MAGE-3 (3), AAGIGILTV and GILTVILGV from Melan-A/MART-1 (4, 29), MLGTHTMEV from gp100 (30) and FLPWHRLFL from tyrosinase (31).

All of the Ags we studied including MAGE-3, Melan-A/MART-1, gp100, tyrosinase, MC1R, and TRP-2 were able to stimulate $CD8^+$ T cell responses in vivo because patients immunized to a vaccine containing these Ags had vaccine-induced responses to peptide epitopes derived from them. There were differences in the frequency with which individual Ags stimulated $CD8^+$ T cell responses, ranging from gp100 which induced $CD8^+$ T cell responses in 27% of the patients to MC1R which induced responses in 9%. However, we could not determine whether this was due to differences in intrinsic immunogenicity in vivo, differences in the relative amount of each Ag present in the vaccine, or a selection bias in the number or type of peptides derived from each Ag selected for testing.

Surprisingly, we did not find any of the peptides to be were clearly immunodominant, as none induced $CD8^+$ T cell responses to a much greater extent than the others. This is in contrast to well-described immunodominance previously reported for one peptide we studied, Melan-A/MART-$1_{27–35}$. The inability to detect frequent immune responses to this particular peptide did not appear to result from lack of sensitivity as we detected responses to the influenza peptide positive control in the majority of patients. Because of its much lower binding affinity than all of the other peptides, it is possible that we would have seen greater recognition had we used more peptide in this specific case or used the decapeptide that has been reported by Romero et al. (32) to have much greater recognition by T cells from different individuals than the nonapeptide.

Our most important finding was the marked HLA-independent heterogeneity in the ability of different patients to develop $CD8^+$ T cell responses to the same peptide or to the same Ag. This was not due to differences in the amount of peptide or Ag used to immunize the patients, since all received the same dose of the same vaccine. Nor was it due to differences in the immunological competence, since all patients were immunologically competent as evidenced by their ability to react to recall Ags and the majority of patients reacted to the control influenza peptide $FluM1_{58–66}$. Nor was it due to differences in the HLA subclass restriction of the peptides or of the patients, since 21 of the 22 patients were $A*0201^+$ and the peptides were all HLA-A*0201-restricted. The one patient who was of the A*0205 subtype did not respond to any peptide. Nor was it due to differences in the solubility or affinity of the peptides used in the assays, since all patients cells were exposed to all of the peptides under the same conditions.

In summary, we have directly demonstrated the ability of seven melanoma-associated Ags to stimulate a $CD8^+$ T cell response in humans and identified multiple $CD8^+$ T cell peptide epitopes on each of these Ags that can be recognized by $CD8^+$ T cells in vivo. These peptides and Ags are attractive candidates for vaccine construction because they can stimulate $CD8^+$ T cell responses. There was marked HLA-independent heterogeneity in the ability of the same peptide or Ag to stimulate $CD8^+$ T cell responses in different patients, and no single peptide or Ag was clearly immunodominant. Regardless of the reasons for this heterogeneity, the implication for cancer vaccine design is that the stimulation of $CD8^+$ T cell responses to cancer will be maximized by constructing vaccines from multiple peptides or multiple Ags.

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