The Journal of Immunology

Positional Scanning-Synthetic Peptide Library-Based Analysis of Self- and Pathogen-Derived Peptide Cross-Reactivity with Tumor-Reactive Melan-A-Specific CTL

Verena Rubio-Godoy, Valérie Dutoit, Yingdong Zhao, Richard Simon, Philippe Guillaume, Richard Houghten, Pedro Romero, Jean-Charles Cerottini, Clemencia Pinilla and Danila Valmori

J Immunol 2002; 169:5696-5707; ;
doi: 10.4049/jimmunol.169.10.5696
http://www.jimmunol.org/content/169/10/5696

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Positional Scanning-Synthetic Peptide Library-Based Analysis of Self- and Pathogen-Derived Peptide Cross-Reactivity with Tumor-Reactive Melan-A-Specific CTL[1]

Verena Rubio-Godoy,[2]* Valérie Dutoit,[2]* Yingdong Zhao,[†] Richard Simon,[†] Philippe Guillaume,[‡] Richard Houghten,[§] Pedro Romero,* Jean-Charles Cerottini,[‡] Clemencia Pinilla,[3§] and Danila Valmori[4]*

Synthetic combinatorial peptide libraries in positional scanning format (PS-SCL) have recently emerged as a useful tool for the analysis of T cell recognition. This includes identification of potentially cross-reactive sequences of self or pathogen origin that could be relevant for the understanding of TCR repertoire selection and maintenance, as well as of the cross-reactive potential of Ag-specific immune responses. In this study, we have analyzed the recognition of sequences retrieved by using a biometric analysis of the data generated by screening a PS-SCL with a tumor-reactive CTL clone specific for an immunodominant peptide from the melanocyte differentiation and tumor-associated Ag Melan-A. We found that 39% of the retrieved peptides were recognized by the CTL clone used for PS-SCL screening. The proportion of peptides recognized was higher among those with both high predicted affinity for the HLA-A2 molecule and high predicted stimulatory score. Interestingly, up to 94% of the retrieved peptides were cross-recognized by other Melan-A-specific CTL. Cross-recognition was at least partially focused, as some peptides were cross-recognized by the majority of CTL. Importantly, stimulation of PBMC from melanoma patients with the most frequently recognized peptides elicited the expansion of heterogeneous CD8$^+$ T cell populations, one fraction of which cross-recognized Melan-A. Together, these results underline the high predictive value of PS-SCL for the identification of sequences cross-recognized by Ag-specific T cells. *The Journal of Immunology,* **2002, 169: 5696–5707.**

T lymphocytes recognize Ag-derived peptides presented on the surface of APC in association with MHC molecules. Specific recognition of the large variety of these complexes is achieved through their interaction with clonally distributed $\alpha\beta$ TCR. The number of different TCR that the immune system is able to generate through a sophisticated recombination process is theoretically large enough to ensure recognition of any potential complex (1). However, several factors limit both the composition and the size of the actual TCR repertoire. Important constraints to its composition are imposed during thymic selection. Immature thymocytes are "educated" by antigenic self-peptide/MHC (pMHC) complexes presented on thymic APC (2). Thymocytes recognizing self complexes with high affinity are deleted, whereas those recognizing them with low affinity are selected to undergo further maturation (3). Thus, recognition of a limited number of self complexes tremendously restricts and shapes the TCR repertoire, with the proportion of positive-selected thymocytes not higher than 5% of the total (4). The size of the TCR repertoire is first limited by the number of T cells present in an animal ($\sim 10^{12}$ in an adult human being). The number of distinct specificities in the TCR repertoire is further limited by the fact that, to mount an appropriate immune response to a given antigenic determinant, a certain number of specific T cells have to be present in the repertoire. Indeed, it has been shown that, in a single individual, many different clonotypes usually contribute to immune responses against an antigenic determinant (5).

Therefore, it is becoming increasingly clear, both from theoretical considerations and experimental evidence, that a high degree of cross-reactivity is a normal feature of the TCR and constitutes an essential characteristic of T cell recognition (6). However, both the molecular nature and the extent of the cross-reactivity between the pool of natural antigenic determinants and the T cell repertoire are not immediately obvious and most likely vary widely for each antigenic determinant and for each TCR. Indeed, even if flexible, T cell recognition remains exquisitely specific and very small changes can profoundly affect it (7). These changes do not necessarily need to be located in TCR contact residues of the antigenic peptide but can be due to more-or-less subtle modifications of the MHC/peptide complex conformation, such as variations in the conformation that the peptide itself adopts in the MHC molecule, or even small peptide-dependent variations of the MHC molecule (8–10). Moreover, as the relevant conformation can be mimicked by apparently completely unrelated ligands, cross-reactivity does not necessarily imply sequence homology (11, 12). As a result of this complexity, it is very difficult to predict whether, and to what extent, one antigenic determinant will cross-react with another.

*Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Lausanne Branch, University Hospital, Lausanne, Switzerland; †Molecular Statistics and Bioinformatics Section, Biometric Research Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; ‡Ludwig Institute for Cancer Research, Lausanne Branch, Lausanne, Switzerland; and §Torrey Pines Institute for Molecular Studies and Mixture Sciences, San Diego, CA 92121

Received for publication June 12, 2002. Accepted for publication September 5, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] V.R.-G. is supported by the Swiss Cancer League Grant SLK 782-2-1999. V.D. is supported by Mixture Sciences (San Diego, CA).

[2] V.R.-G. and V.D. contributed equally to this work.

[3] Address correspondence about the use of combinatorial peptide libraries and biometric analysis to Dr. Clemencia Pinilla, Torrey Pines Institute for Molecular Studies and Mixture Sciences, 3550 General Atomics Court, San Diego, CA 92121. E-mail address: cpinilla@tpims.org

[4] Address correspondence and reprint requests to Dr. Danila Valmori, Division of Clinical Onco-Immunology, Hôpital Orthopédique, Avenue Pierre-Decker 4, CH-1005 Lausanne, Switzerland. E-mail address: danila.valmori@inst.hospvd.ch

0022-1767/02/$02.00

An empirical approach for the identification of antigenic determinants cross-recognized by Ag-specific T cells is based on the analysis of the recognition by clonal T cells of synthetic combinatorial peptide libraries in positional scanning format (PS-SCL[5]; Ref. 13). We have recently elaborated a biometric analysis that uses the data generated by screening PS-SCL with T cell clones to analyze and rank all peptides in public protein databases for sequences with predicted reactivity (14). In this study, we have analyzed the recognition by tumor-reactive CTL specific for an immunodominant peptide from the melanocyte differentiation and tumor-associated Ag Melan-A (15), of peptides retrieved in proteins of self or pathogen origin by using biometric analysis of the data generated by screening of PS-SCL with a Melan-A-specific clone.

Materials and Methods

Scoring matrix and database searches

A scoring matrix was generated by using data previously obtained by screening an amidated C terminus decapeptide PS-SCL (16) with the Melan-A-specific CTL clone LAU 203/1.5 in a functional chromium-release assay. A Z-scoring matrix was generated using the average and SD of the percentage of specific-lysis values of multiple experimental data obtained for each mixture defined with one of the 20 L-amino acids in each of the 10 positions of the decamer library (14). Based on the assumption of independent and additive contribution of the individual amino acids at each position of a peptide to the peptide's activity, the score of each individual peptide was calculated by adding individual stimulatory values of the composing amino acids. A program was designed to use the matrix to score all overlapping decapeptides contained in the GenPept protein database (ftp://ftp.ncicfr.gov/pub/genpept) and thus identify sequences with the highest predicted stimulatory scores (14).

Cells

The Melan-A-specific CTL clone LAU 203/1.5 was derived from tumor-infiltrated lymph node cells of patient LAU 203 by limiting dilution culture in the presence of irradiated allogeneic PBMC, PHA, and human (h)rIL-2 (150 IU/ml; Glaxo, Geneva, Switzerland; kindly provided by Dr. M. Nabholz, Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland) as previously described (17). Cells were subsequently expanded by periodic (3- to 4-wk) restimulation in microtiter plates together with irradiated feeder cells in the presence of PHA and hrIL-2. Polyclonal Melan-A monospecific CTL lines and other Melan-A specific clones were similarly generated from Melan-A multimer$^+$-sorted cells as previously described (18). All selected populations were able to specifically lyse Melan-A-expressing melanoma tumor cells. For peptide stimulation of PBMC, CD8$^+$ lymphocytes were positively selected by magnetic cell sorting from PBMC of A2 melanoma patients using a miniMACS device (Miltenyi Biotec, Sunnyvale, CA). Cells of the CD8$^-$ fraction were irradiated (3000 rad) and used as autologous APC. CD8$^+$ highly enriched lymphocytes (1×10^6) were stimulated with peptide (1 μg/ml) and irradiated autologous APC in 2 ml of IMDM (Life Technologies, Basel, Switzerland) containing 8% pooled human serum, hrIL-2 (100 IU/ml), and hrIL-7 (10 ng/ml, R&D Systems, Oxon, U.K.). Cells underwent an additional cycle of stimulation with peptide-pulsed APC before analysis.

Ag-recognition assay

Ag recognition was assessed in chromium-release experiments. Briefly, target cells were labeled with ^{51}Cr for 1 h at 37°C and washed three times. Labeled target cells (1000 cells/well) were incubated in the presence of peptide libraries (100 μg/ml final) or, in peptide titration experiments, with various peptide doses, for 15 min at room temperature before the addition of effector cells at a lymphocyte-to-target cell ratio of 10:1. Chromium release was measured in supernatants harvested after 4-h incubation at 37°C. The percentage of specific lysis was calculated as: $100 \times$ [(experimental $-$ spontaneous release)/(total $-$ spontaneous release)]

Multimers, mAbs, and flow cytometry analysis

A2/peptide multimers incorporating the Melan-A$_{26-35}$ A27L analog (8) or peptides MSI-44–10 (PT 178–187), MSI-44–25 (PV 454–463), and MSI-44–56 (CT 51–60) were synthesized as described previously (19). Cells were stained with multimers^{PE} (4.5 μg/ml in 20 μl) for 1 h at room temperature, before the addition of anti-CD8^{FITC} mAb (BD Biosciences, San Jose, CA) and further incubated for an additional 30 min at 4°C. At the end of the incubation period, cells were washed and analyzed by flow cytometry on a FACScan (BD Biosciences). Data analysis was performed using the CellQuest software.

[5] Abbreviations used in this paper: PS-SCL, synthetic combinatorial peptide libraries in positional scanning format; h, human; A2, HLA-A*0201

Results

Recognition of self- and pathogen-derived peptides selected on the basis of their potential cross-reactivity with Melan-A by clone LAU 203/1.5

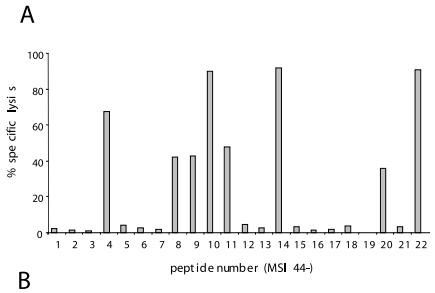
In a previous study, we used the Melan-A-specific and tumor-reactive CTL clone LAU 203/1.5 to screen an amidated C terminus decapeptide PS-SCL, (16) in a functional chromium release assay (CTL assay). Based on those results, in this study, we performed a biometric data analysis (as recently reported (14) and further detailed in *Materials and Methods*) that allows the use of the results of the PS-SCL screening to search for potentially cross-reactive sequences in public protein databases. As illustrated in Table I, our search retrieved 21 peptides of self origin and 25 peptides of viral origin with higher predicted T cell stimulatory values (scores) than the parental Melan-A$_{26-35}$ decapeptide (MSI-44–22, Table I). These peptides were selected for analysis. Among peptides of bacterial origin, a high number (280) scored higher than Melan-A$_{26-35}$. Of those, the first 25 peptides were selected for analysis. For consistency with the PS-SCL used for the screening, peptides were synthesized with amidated C termini. Recognition of the selected peptides by the CTL clone LAU 203/1.5 was initially tested at a single peptide dose (1 μg/ml) in a CTL assay. An example of the data obtained is illustrated in Fig. 1*A*. Data obtained for all peptides tested and a summary thereof are reported in Fig. 1, *B* and *C*, respectively. Specific lysis of $>10\%$ was considered significant, because values $<10\%$ were consistently obtained for a group of 100 unrelated peptides similarly tested (data not shown). Thirty-nine percent of the selected peptides were significantly recognized by clone LAU 203/1.5 (Fig. 1*C*). It is noteworthy that the proportion of peptides recognized was higher among those of bacterial origin than those of self or viral origin. The relative efficiency of recognition of the selected peptides was further assessed by performing peptide titrations as illustrated in Fig. 2*A*. Results are summarized in Fig. 2*B*. Of the 28 peptides recognized by clone LAU 203/1.5, 12 were recognized with roughly comparable efficiency (relative recognition <10 but >0.1), as compared with Melan-A $_{26-35}$; 4 were recognized significantly more efficiently (relative recognition of ≥ 10); and the remaining 12 were recognized significantly less efficiently (relative recognition of ≤ 0.1). Cross-reactivity between amidated and free C termini peptides was tested for a group of peptides selected among the most active ones. As summarized in Fig. 2*C* and consistent with our previous data (20), peptides bearing free C termini were generally recognized as well as or more efficiently than their amidated C termini counterparts, although to a variable extent.

Recognition of PS-SCL-retrieved peptides is more frequent among predicted A2 binders of higher affinity than Melan-A$_{26-35}$ and among peptides with the highest scores

To elucidate the factors impacting on the recognition of the selected peptides by clone LAU 203 1.5, their potential binding capacity to A2 was first determined using an algorithm that calculates the theoretical dissociation rate of the corresponding A2/peptide complexes based on their amino acid composition (available at the Bioinformatics and Molecular analysis section of the National Institutes of Health website, http://bimas.dcrt.nih.gov/

Table I. *Self- and pathogen-derived peptides selected on the basis of their potential crossreactivity with Melan-A*

Peptide	Score	Species	Protein	Location
MSI 44-human				
1	161.45	*Homo sapiens*	KIAA0935	370–379
2	159.82	*H. sapiens*	MRP3	1353–1362
3	159.79	*H. sapiens*	Hypothetical 32.3-kDa protein	150–159
4	159.07	*H. sapiens*	Thioredoxin reductase GRIM-12	465–474
5	158.35	*H. sapiens*	SLC1A1 or EAAT3 or EAAC1	289–298
6	156.17	*H. sapiens*	Adenylate kinase isoenzyme 2	137–146
7	155.54	*H. sapiens*	Programed cell death protein 8	98–107
8	155.3	*H. sapiens*	Integrin α_{11}	991–1000
9	154.83	*H. sapiens*	P47	317–326
10	154.74	*H. sapiens*	PG transporter	178–187
11	154.54	*H. sapiens*	ABC transporter MOAT-C	1257–1266
12	154.41	*H. sapiens*	KIAA0735	206–215
13	154.25	*H. sapiens*	Hypothetical 20-kDa protein	3–12
14	154.11	*H. sapiens*	Endothelin-1 receptor	192–201
15	153.82	*H. sapiens*	G-protein-coupled receptor RE2	364–373
16	153.74	*H. sapiens*	MRP3	1353–1362
17	153.21	*H. sapiens*	IGHG1	103–112
18	152.61	*H. sapiens*	Monocarboxylate transporter 8	253–262
19	152.15	*H. sapiens*	PhosphorylaseB kinase α reg. chain	1136–1145
20	151.97	*H. sapiens*	Hypothetical protein KIAA0274	115–124
21	151.89	*H. sapiens*	MRP3	1353–1362
22	151.36	*H. sapiens*	Melan-A/Mart-1	26–35
Viral				
23	161.81	HSV	Capsid protein p40	235–244
24	161.06	Little cherry closterovirus	MT and HEL domains	1684–1693
25	160.47	Pseudorables virus	Glycoprotein GIII	454–463
26	159.52	Pseudorables virus	Glycoprotein C	462–471
27	158.4	Human rotavirus	Outer capsid protein VP4	443–452
28	158.13	Human rotavirus	Outer capsid protein VP5	443–452
29	157.76	Canine calicivirus	Capsid protein	339–348
30	156.07	Variola virus	(*XHOI-F*, *O*, *H*, *P*, *Q*) genes	272–281
31	154.75	Bovine herpesvirus type 1	Capsid protein p40	242–251
32	154.58	Bovine herpesvirus 2	DNA-dependent DNA polymerase	608–617
33	154.36	Vaccinia virus	Protein A49	146–155
34	154.35	Hepatitis E virus	Nonstructural polyprotein	255–264
35	154.18	Tobacco necrosis virus	RNA-directed RNA polymerase	239–248
36	153.64	Gallid herpesvirus 1	UL22 product homolog	268–277
37	153.57	Puma lentivirus 14	GAG polyprotein	920–929
38	152.86	Murine CMV	Helicase/primase complex protein	653–664
39	152.75	Human CMV	Hypothetical protein HVLF2	47–56
40	152.68	Mumps virus	Fusion glycoprotein	102–111
41	152.64	Human parainfluenza virus 1	L protein	1683–1692
42	152.19	SINP virus	Hypothetical 36.5-kDa protein	87–96
43	151.81	Rotavirus G8	Outer capsid protein VP4	443–452
44	151.74	Mouse CMV 1	M83 protein	320–329
45	151.59	TT virus	Hypothetical 15.9-kDa protein	87–96
46	151.5	Human adenovirus type 12	DNA terminal protein	579–588
47	151.49	*Nilaparvata lugens* reovirus	23.6 kDa putative nonstructural protein	74–83
Bacterial				
48	180.19	*Chlorobium tepidum*	Hypothetical 22.8-kDa protein	150–159
49	175.48	*Bacillus subtilis*	YKOR	431–440
50	173.12	*B. subtilis*	Putative transporter	260–269
51	171.51	*Deinococcus radiodurans*	Amino transferase class I	267–276
52	170.17	*B. subtilis*	Hypothetical 48.9-kDa protein	264–273
53	170.06	*Rickettsia prowazekii*	Cell division protein FTSK homolog	190–199
54	168.15	*Propionigenium modestum*	ATP synthase β-chain	327–336
55	167.6	*Aeromonas salmonicida*	EPSP synthase	262–271
56	167.22	*Chlamydia trachomatis*	Arginine/ornithine antiporter	51–60
57	166.51	*Nostoc punctiforme*	C-terminal protease	197–206
58	166.35	*Streptomyces coelicolor*	Putative secreted protein	669–678
59	166.16	*Escherichia coli*	Hypothetical 74.9-kDa protein	457–466
60	165.62	*Synechococcus sp.*	REPA	65–74
61	164.87	*Bacillus pumilus*	Anthranylate synthase componant 1	59–68
62	164.77	*Methylobacterium Extorquens*	Hypothetical protein in PHAC 3′ region	37–46
63	163.99	*Salmonella typhimurium*	LTKB homolog	180–189
64	163.22	*Burkholderia cepacia*	Hypothetical 23.3-kDa protein	158–167
65	163.1	*Aquifex geolicus*	ATP synthase β-chain	339–348
66	163.07	*E. coli*	Colicin V secretion ATP-binding protein CVAI	465–474
67	162.94	*Helicobacter pylori J99*	Serine acetyltransferase	61–70
68	162.82	*E. coli*	ATP-dependent protease LA	751–760
69	162.52	*Azospirillum brasiliense*	Glutamate synthase (NADPH) large chain	781–790
70	162.36	*Rhizobium meliloti*	RHSC protein	143–152
71	162.18	*Rhizoblum sp.*	Y4FN probable ABC transporter permease	452–461
72	162.17	*E. coli*	K^+/H^+ antiporter	36–45



B

		Human			Viral			Bacterial		
MSI 44-	1	R V T D E A G H P V	-	23	R Q A G I A G H T Y	-	48	R L S G H G I H P V	++++
	2	N V A D I G L H D V	-	24	R V S N I A I A T G	-	49	R V A S I G L H P S	++++
	3	R I S G V C I H P R	-	25	V I A G I G I L A I	++++	50	V M A G I A I F P A	++++
	4	L D S T I G I H P V	++++[a]	26	V I A G I A I L A I	-	51	R V A G L G G H P S	-
	5	V L T G L A I H S I	-	27	R L S G I Y G L P A	-	52	L L A G L A I F P A	-
	6	R I T G R L I H P K	-	28	R M S G I Y G L P A	-	53	R L S L I G L F P I	++++
	7	R I S G L G L T P E	-	29	N T T D I G I H V V	++++	54	R I A S L G I Y P A	++++
	8	R I Q N L G L F P I	+++	30	M I A G I G I S L I	++++	55	R V T G I G K H S I	-
	9	R I S D I R L F I V	+++	31	R E A G I A G H T Y	-	56	M L S G I G I F F I	++++
	10	L L A G I G T V P I	++++	32	R L A G I G L T R A	+	57	T R A N I A I H P V	++
	11	R I S D I G L A D L	+++	33	R I A D I D I K Q V	-	58	V L S S I G I F P I	++++
	12	L I S G I G I G G A	-	34	R V R G I G C H F V	-	59	R V S R I A V H P A	+
	13	R I S A I I L H P N	-	35	R Y S G I G G H L L	-	60	R V T G I G L L T G	++++
	14	R V Q G I G I P L V	++++	36	R I S D L A L N F I	++++	61	R Y S F I G L H P F	-
	15	R I T D L G L S P H	-	37	R I T G I C F H F G	-	62	L V A G I A I G P F	+
	16	N V A D I G L H D L	-	38	R L A G I L D H T L	-	63	R I A D I P I F I I	-
	17	R L S E L A I F G V	-	39	R I A G L L L F Q I	-	64	S I A D I A I Y P W	-
	18	A V A F I G L H T S	-	40	R F A G I A I G I A	-	65	R L A E L G I Y P A	++++
	19	M L A D I E I H S I	-	41	R V R G I G I P E V	++++	66	R I A D I A L H E K	-
	20	K M A D I G G H A I	++	42	C T S I I G I F P V	+++	67	F I T G I E I H P G	-
	21	N V A D I G F H D V	-	43	R I S G L Y G L P A	-	68	V I A D L D I H P V	-
	22	E A A G I G I L T V	++++	44	L I P G L D I H P S	-	69	R I S G I G L N G I	++++
				45	R A P S I G I L P A	++++	70	L I A G H G I H P C	-
				46	R Q A D I P L P P L	-	71	R S A F I G I D P A	-
				47	N F A G I A I L F I	-	72	L L A G I A I G P W	-

a:'+': >10% specific lysis, '++': >20% specific lysis, '+++': >40 % specific lysis, '++++': >60% specific lysis

C

Peptide origin	Total	Recognized [a]	Percent [b]
Human	21	7	33
Viral	25	8	32
Bacter ial	25	13	52
Total	71	28	39

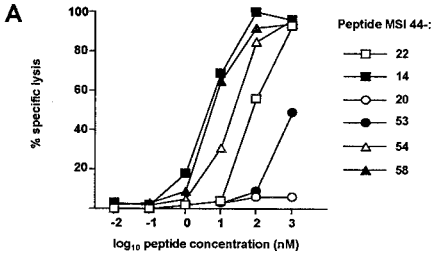
a: number of peptides recognized
b: percentage of peptides recognized

FIGURE 1. Recognition of peptides retrieved by biometric data analysis of PS-SCL screening with clone LAU 203/1.5. Peptide recognition by clone LAU 203/1.5 was assessed in a CTL assay by using T2 cells as targets. Recognition was tested at a single peptide dose (1 μg/ml). *A*, Example of the data obtained for the group of peptides of self origin. *B*, Data obtained for all peptides of self, viral, and bacterial origin. *C*, Summary of the peptides recognized in each group.

molbio/hla_bind/) (21). As expected from both the absence of canonical "anchor residues" and our previous experimental data (8, 17, 22), peptide Melan-A_{26-35} was predicted as a relatively poor binder (theoretical $t_{1/2}$ of β_2m dissociation was around 3300-fold lower than for a known good A2 binder, peptide Flu-MA_{58-66}, supplemental data, Table II). Among the selected peptides, 35% had a relative predicted binding lower than that of Melan-A_{26-35}, whereas the majority (65%) had a similar or higher (up to >20,000-fold) predicted binding value. Interestingly, the frequency of peptides recognized by clone LAU 203/1.5 was high (89%; Fig. 3*A*) among peptides with relative predicted binding equal to or greater than that of Melan-A_{26-35}. Indeed, only 13% of peptides with predicted binding lower than that of Melan-A_{26-35} were significantly recognized, whereas about half (47%) of peptides with predicted binding equal to or greater than that of Melan-A_{26-35} were significantly recognized (Fig. 3*A*). In addition, all of the peptides that were recognized more efficiently than the parental Melan-A peptide had a higher predicted A2 binding value (Fig. 3*B*). Relative predicted binding was independent from peptide score (the mean score was similar in the two groups, Fig. 3*C*), consistent with the peptide selection procedure used that was, in this sense, completely unbiased. However, when peptides were divided into two groups according to whether their scores were higher or lower than the mean score of all peptides tested (Fig. 3*D*), ~70% of the peptides in the high scoring group were recognized. In addition, 75% of the peptides that were recognized more efficiently than the parental Melan-A peptide belonged to the group with the highest scores (Fig. 3*E*). To compare predicted to experimental binding, experimental binding was assessed in a functional competition assay as described previously (17) for peptides available in the terminal C-free form (Fig. 2*C*). The experimental values obtained were consistent with predicted binding values (not shown).

A large proportion of PS-SCL-retrieved peptides are cross-recognized by other Melan-A-specific CTL

We next investigated whether cross-recognition of the selected peptides was found only for clone LAU 203/1.5 or also for other Melan-A-specific CTL. To this end, we tested their recognition by a large panel of Melan-A-monospecific CTL lines (18) and clones derived from four melanoma patients (LAU 203, LAU 337, LAU 455, and LAU 465) and one healthy donor (HD 421). Our previous studies of the TCR repertoire of Melan-A-specific CTL have shown that the latter is extremely large and diverse and mostly nonoverlapping between different individuals (5, 23). Thus, the majority of Melan-A-specific CTL clones used in this study are



B

Peptide origin [a]	MSI 44-	Relative activity [b]
Human	22	1 [c]
	4	0.2
	8	0.07
	9	< 0.07
	10	15
	11	0.1
	14	16
	20	< 0.07
Viral	25	0.1
	29	0.2
	30	< 0.07
	32	< 0.07
	36	0.7
	41	0.2
	42	< 0.07
	45	0.7
Bacterial	48	3
	49	3
	50	4
	53	0.08
	54	5
	56	146
	57	< 0.07
	58	11
	59	< 0.07
	60	0.2
	62	< 0.07
	65	4
	69	0.3

a: peptide titration were performed for peptides giving more than 10% specific lysis at a concentration of 1 µg/ml (Figure 1B).
b: the relative activity of each peptide was calculated as the [nM] of peptide Melan-A_{26-35} giving 50% maximal activity divided by the corresponding value for the test peptide.
c: the 50% maximal activity for peptide Melan-A_{26-35} was obtained at 70 nM

C

	Peptide origin	Relative recognition[a]
Human	Melan-A 26-35	107
	Prostaglandin transporter 178-187	3
	Endothelin-1 Receptor 192-201	65
Viral	Pseudorabies virus, Glycoprotein GIII 454-463	25
	Canine calicivirus, Capsid protein 339-348	0.6
Bacterial	Chlorobium tepidum, Hyp. 22.8 kD prot. 150-159	0.4
	Bacillus subtilis, YKOR 431-440	4
	Bacillus subtilis, Put. Transporter 260-269	17
	Propionigenium modestus, ATP synth. beta-chain 327-336	3
	Chlamydia trachomatis, Arg./orn. antiporter 51-60	6
	Streptomyces coelicolor, Put. secr. prot. 669-678	10
	Aquifex geolicus, ATP synth. beta-chain 338-348	0.7

a: Recognition of free C-termini peptides relative to the corresponding NH_2 C-termini forms. Peptide titrations were performed in parallel for free C-termini peptides and the corresponding NH_2 C-termini forms. Based on these data, the relative activity of each free C-terminus peptide was calculated relative to that of the corresponding NH_2 C-terminus form by dividing the [nM] of the NH_2 C-terminus peptide giving 50% maximal activity by the corresponding value obtained for the free C-terminus form.

FIGURE 2. Efficiency of recognition of retrieved peptides and comparison between NH_2 and free C termini peptides. *A*, Efficiency of peptide recognition was determined by assessing specific lysis in the presence of graded peptide concentrations. *B*, Summary of the results. *C*, Recognition of free C termini peptides relative to their NH_2 C termini counterparts.

Table II. *Predicted A2 binding for PS-SCL-retrieved peptides*

MSI 44-	Species	Protein	Location	Predicted Binding[a]	Relative Predicted Binding[b]
Human					
1	*H. sapiens*	KIAA0935	370–379	3.244	19.4
2	*H. sapiens*	MRP3	1353–1362	57.389	343.6
3	*H. sapiens*	Hypothetical 32.3-kDa protein	150–159	0.005	0.03
4	*H. sapiens*	Thioredoxin reductase GRIM-12	465–474	0.378	2.3
5	*H. sapiens*	SLC1A1 or EAAT3 or EAAC1	289–298	40.792	244.3
6	*H. sapiens*	Adenylate kinase isoenzyme 2	137–146	0.005	0.03
7	*H. sapiens*	Programed cell death protein 8	98–107	0.005	0.03
8	*H. sapiens*	Integrin α_{11}	991–1000	19.136	114.6
9	*H. sapiens*	P47	317–326	214.455	1284.2
10	*H. sapiens*	PG transporter	178–187	17.736	106.2
11	*H. sapiens*	ABC transporter MOAT-C	1257–1266	27.699	165.9
12	*H. sapiens*	KIAA0735	206–215	0.347	2.1
13	*H. sapiens*	Hypothetical 20-kDa protein	3–12	0.024	0.1
14	*H. sapiens*	Endothelin-1 receptor	192–201	13.997	83.8
15	*H. sapiens*	G-protein-coupled receptor RE2	364–373	0.097	0.6
16	*H. sapiens*	MRP3	1353–1362	17.627	105.6
17	*H. sapiens*	IGHG1	103–112	3804.077	22778.9
18	*H. sapiens*	Monocarboxylate transporter 8	253–262	0.015	0.1
19	*H. sapiens*	PhosphorylaseB kinase α reg. chain	1136–1145	167.248	1001.5
20	*H. sapiens*	Hypothetical protein KIAA0274	115–124	108.124	647.4
21	*H. sapiens*	MRP3	1353–1362	47.408	283.9
22	*H. sapiens*	Melan-A/Mart-1	26–35	0.167	1.0
Viral					
23	HSV	Capsid protein p40	235–244	0.008	0.05
24	Little cherry closterovirus	MT and HEL domains	1684–1693	0.015	0.1
25	Pseudorabies virus	Glycoprotein GIII	454–463	5.609	33.6
26	Pseudorabies virus	Glycoprotein C	462–471	5.609	33.6
27	Human rotavirus	Outer capsid protein VP4	443–452	4.968	29.7
28	Human rotavirus	Outer capsid protein VP5	443–452	3.588	21.5
29	Canine calicivirus	Capsid protein	339–348	3.735	22.4
30	Variola virus	(*XHOI-F*, *O*, *H*, *P*, *Q*) genes	272–281	5.609	33.6
31	Bovine herpesvirus type 1	Capsid protein p40	242–251	0.001	0.001
32	Bovine herpesvirus 2	DNA-dependent DNA polymerase	608–617	11.426	68.4
33	Vaccinia virus	Protein A49	146–155	90.183	540
34	Hepatitis E virus	Nonstructural polyprotein	255–264	2.544	15.2
35	Tobacco necrosis virus	RNA-directed RNA polymerase	239–248	0.003	0.02
36	Gallid herpesvirus 1	UL22 product homolog	268–277	74.401	445.5
37	Puma lentivirus 14	GAG polyprotein	920–929	0.107	0.6
38	Murine CMV	Helicase/primase complex protein	653–664	21.362	127.9
39	Human CMV	Hypothetical protein HVLF2	47–56	19.136	114.6
40	Mumps virus	Fusion glycoprotein	102–111	0.000001	<0.001
41	Human parainfluenza virus 1	L protein	1683–1692	1.064	6.4
42	Spodoptera littoralis nuclear polyhedrosis virus	Hypothetical 36.5-kDa protein	87–96	12.886	77.2
43	Rotavirus G8	Outer capsid protein VP4	443–452	0.683	4.1
44	Mouse CMV 1	M83 protein	320–329	0.04	0.2
45	TT virus (hepatitis TT)	Hypothetical 15.9-kDa protein	87–96	0.159	1.0
46	Human adenovirus type 12	DNA terminal protein	579–588	20.425	122.3
47	*Nilaparvata lugens* reovirus	23.6-kDa putative nonstructural protein	74–83	0.092	0.6
Bacterial					
48	*C. tepidum*	Hypothetical 22.8-kDa protein	150–159	20.796	124.5
49	*B. subtilis*	YKOR	431–440	0.015	0.1
50	*B. subtilis*	Putative transporter	260–269	81.369	487.2
51	*D. radiodurans*	Amino transferase class I	267–276	0.007	0.04
52	*B. subtilis*	Hypothetical 48.9-kDa protein	264–273	112.664	674.6
53	*R. prowazekii*	Cell division protein FTSK homolog	190–199	139.174	833.4
54	*P. modestum*	ATP synthase β-chain	327–336	1.571	9.4
55	*A. salmonicida*	EPSP synthase	262–271	0.913	5.5
56	*C. trachomatis*	Arginine/ornithine antiporter	51–60	1301.273	7792.1
57	*N. punctiforme*	C-terminal protease	197–206	0.022	0.1
58	*S. coelicolor*	Putative secreted protein	669–678	236.595	1416.7
59	*E. coli*	Hypothetical 74.9-kDa protein	457–466	1	6.0
60	*Synechococcus sp.*	REPA	65–74	0.015	0.1
61	*B. pumilus*	Anthranylate synthase component 1	59–68	0.000001	<0.001
62	*Methylobacterium Extorquens*	Hypothetical protein in PHAC 3′ region	37–46	0.003	0.02
63	*S. typhimurium*	LTKB homolog	180–189	32.168	192.6
64	*B. cepacia*	Hypothetical 23.3-kDa protein	158–167	0.097	0.6
65	*A. geolicus*	ATP synthase β-chain	339–348	46.848	280.5
66	*E. coli*	Colicin V screstion APT-binding protein CVAB	465–474	0.019	0.1
67	*H. pylori J99*	Serine acetyltransferase	61–70	0.108	0.6
68	*E. coli*	ATP-dependent protease LA	751–760	153.311	918.0
69	*A. brasiliense*	Glutamate synthase (NADPH) large chain	781–790	3.299	19.8
70	*R. meliloti*	RHSC protein	143–152	2.671	16.0
71	*Rhizobium sp.*	Y4FN probable ABC transporter permease	452–461	0.037	0.2
72	*E. coli*	K^+/H^+ antiporter	36–45	0.038	0.2
	Influenza virus	Matrix protein	58–66	551	3299.4

[a] The theoretical A2 binding capacity of retrieved peptides was evaluated using an algorithm available at Bioinformatics and Molecular analysis section of the National Institutes of Health website (http://bimas.dcrt.nih.gov/molbio/hla_bind/). Numbers correspond to the theoretical dissociation rate of A2/peptide complexes calculated based on their amino acid composition.

[b] Relative predicted binding was calculated for each peptide as the predicted binding value for the corresponding peptide divided by the predicted binding value for the Melan-$A_{26–35}$.

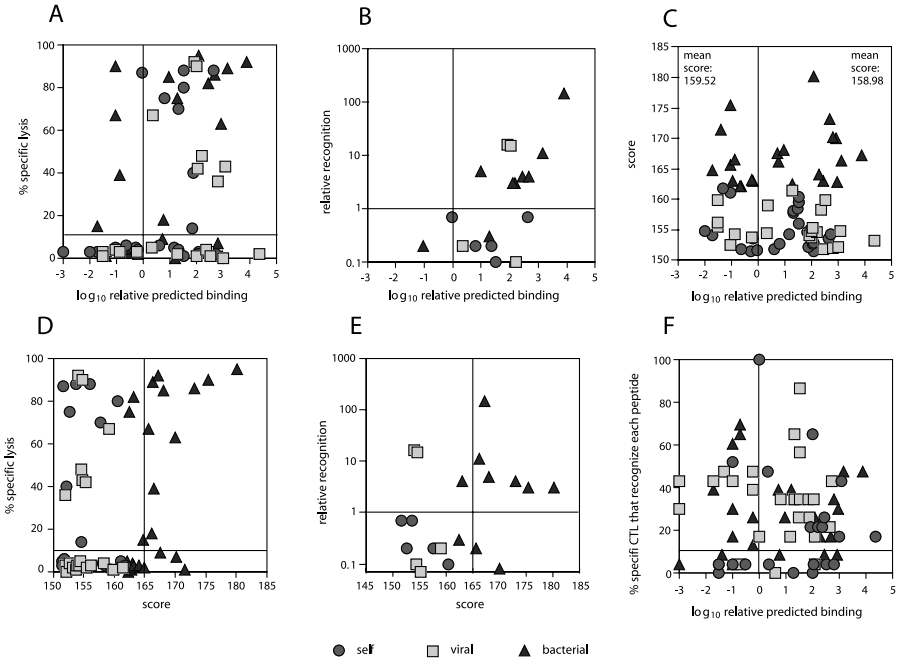


FIGURE 3. Correlation between peptide recognition, predicted A2 binding, and predicted stimulatory scores of retrieved sequences. *A*, Predicted A2 binding of synthetic peptides corresponding to retrieved sequences, calculated relative to peptide Melan-A_{26-35} (bar in the *x*-axis), is plotted against the percentage of specific lysis obtained for each peptide at a peptide concentration of 1 μg/ml. Specific lysis values were considered significant if >10% (bar in the *y*-axis). *B*, Relative predicted binding is plotted against the efficiency of recognition of each peptide (calculated as in Fig. 2*B*). *C*, Relative predicted binding is plotted against peptide score. *D*, Peptide score is plotted against specific lysis (as in *A*). *E*, Peptide score is plotted against relative recognition (as in *B*). *F*, Relative predicted binding is plotted against percentage of specific CTL that recognize each peptide (Table III).

likely to express different TCR. In addition, TCR sequences were previously determined in the case of CTL clones from LAU 337 (24) and HD 421 (25), and all were found to be distinct. Results obtained are shown in Table III and summarized in Tables IV and V. Surprisingly, we found a degree of cross-reactivity that was higher than that of clone LAU 203/1.5. Indeed, the proportion of peptides recognized by at least one of the CTL populations analyzed accounted for 94% of the total (Table IV). Melan-A-specific clones largely differed in terms of their ability to cross-recognize the peptides, ranging from no detectable cross-recognition by clone LAU 337/6C3 to recognition of 68% (48/71) of the peptides by clone LAU 337/2A5 (Table V). Polyclonal monospecific lines generally displayed a higher degree of cross-recognition as compared with CTL clones, possibly because of the combined reactivity of clonal populations composing these lines. The polyclonal monospecific population derived from patient LAU 203 cross-recognized a higher proportion of peptides (68%) than did clone LAU 203/1.5. It is of note that, in contrast with the data obtained for clone LAU 203/1.5 (Fig. 3*A*), there was no correlation between the ability of the peptides to be recognized by different Melan-A-specific CTL and their A2-binding capacity (Fig. 3*F*). Interestingly, some peptides were recognized by the majority of CTL (i.e., MSI-44–25, Table IV). To further explore the extent of cross-reactivity between these peptides (in particular those most frequently recognized by Melan-A-specific CTL) and Melan-A, we synthesized A2/peptide multimers incorporating three of the most frequently recognized peptides, one of self origin (peptide PT 178–187 from PG transporter), one of viral origin (peptide PV 454–463 from the glycoprotein GIII precursor of pseudorabies virus), and one of bacterial origin (peptide CT 51–60 from the arginine/ornithine antiporter of *Chlamydia trachomatis*). These multimers specifically stained clone LAU 203/1.5 but not clones of unrelated specificity; in addition, they were able to specifically stain ex vivo a significant proportion of circulating $CD8^+$ T cells from patient LAU 203, similar to what had been observed previously with multimers incorporating a Melan-A_{26-35} analog A27L (Ref. 26 and data not shown).

Melan-A-related peptides frequently recognized by specific CTL are immunogenic in vitro and elicit heterogeneous CTL responses partially cross-reactive with Melan-A

To determine whether the peptides frequently cross-recognized by Melan-A-specific CTL could elicit CTL responses cross-reactive with Melan-A, we used three of the most frequently cross-recognized peptides to stimulate in vitro highly enriched $CD8^+$ T lymphocytes from melanoma patients. As internal controls, we included $CD8^+$ T cells cultured under the same conditions, but in the absence of any exogenously added peptide, or stimulated with Melan-A_{26-35} parental peptide or with the analog Melan-A_{26-35} A27L. Consistent with our previous data (27), stimulation with the analog Melan-A_{26-35} A27L elicited a specific response significantly higher than that observed with the Melan-A_{26-35} parental peptide for all patients (Fig. 4, *A* and *B*). Importantly, as illustrated in Fig. 4*A* for patient LAU 241, each of the three selected peptides elicited an even stronger response when tested with multimers incorporating the peptide used for in vitro stimulation (autologous multimers), whereas a lower, but very significant, proportion of $CD8^+$ Melan-A_{26-35} A27L multimer$^+$ cells were detected in each of these cultures. Similar results were obtained in the case of two additional melanoma patients (Fig. 4*B*) and two healthy donors (not shown). To further dissect the heterogeneity of the CTL response elicited by Melan-A related peptides, $CD8^+$ autologous multimer$^+$ cells from each culture were isolated by cell sorting, expanded in vitro with PHA, and stained again with multimers incorporating either the autologous peptide or peptide Melan-A_{26-35} A27L. In each case, the resulting populations were homogeneously stained by autologous multimers, whereas only part of these cells were stained by multimers incorporating peptide Melan-A_{26-35} A27L (Fig. 5*A*). $CD8^+$ Melan-A_{26-35} A27L multimer$^+$ and multimer$^-$ cell fractions were further isolated from each culture by cell sorting, and their activity was analyzed in CTL assay. As illustrated in Fig. 5*B* for one of the cultures and summarized in Fig. 5*C* for all tested cultures from two melanoma patients, $CD8^+$ Melan-A_{26-35} A27L multimer$^+$ fractions cross-recognized peptide Melan-A_{26-35} A27L much more efficiently

Table III. *Recognition of MSI 44 peptides by Melan-A-specific CLT*[a]

	Melan-A-Monospecific Polyclonal CTL Lines								Melan-A-Specific CTL Clones			
MSI 44-	LAU 203	LAU 181	HD 421	LAU 455	LAU 337	LAU 52	LAU 465	MSI 44-	LAU 203/1.5	LAU 203/17	LAU 203/1.3	HD 421 2/4A12
Human								Human				
1	–	–	–	–	–	–	–	1	–	–	–	–
2	–	–	–	–	–	–	–	2	–	–	–	–
3	–	–	–	–	–	–	–	3	–	–	–	–
4	++	–	–	–	–	–	–	4	++++	–	–	–
5	–	–	–	–	–	–	+	5	–	–	–	+
6	–	–	–	–	–	–	–	6	–	–	–	–
7	–	–	–	–	–	–	–	7	–	–	–	–
8	–	–	–	–	–	–	–	8	+++	–	–	–
9	+++	+	+++	–	–	++	++	9	+++	–	–	+
10	++++	++	++++	++	–	++	+++	10	++++	+	–	++++
11	++	–	++	–	–	++	++	11	+++	–	–	–
12	+++	++++	+++	–	+	++	+++	12	–	–	+	–
13	–	–	–	–	+	–	–	13	–	–	–	–
14	+++	–	–	–	+	–	+	14	++++	–	–	–
15	–	–	–	–	–	–	–	15	–	–	–	++
16	–	–	–	–	–	–	–	16	–	–	–	–
17	–	–	–	–	+	–	+	17	–	–	–	–
18	++	+	+	–	++	+	++	18	–	++	–	++
19	–	–	–	–	++	–	+	19	–	–	–	–
20	–	–	–	–	+	–	–	20	++	–	–	–
21	–	–	+	–	+	+	++	21	–	+++	–	–
22	++++	++++	++	+++	+++	++++	+++	22	++++	++++	++	++++
Viral								Viral				
23	–	–	–	–	++	++	++	23	–	+	–	+
24	++	–	+	–	+	+	++	24	–	–	–	+
25	++++	++++	++	+++	+++	++	++++	25	++++	++++	+++	++++
26	++	–	–	–	+	–	++	26	–	+	–	+
27	–	+	–	–	++	–	+	27	–	–	–	–
28	+	–	–	–	++	+	+	28	–	–	–	+
29	++	++++	++	+	++	++	+++	29	++++	+	+++	–
30	++++	++++	+++	–	–	+++	++++	30	++++	++++	–	++++
31	++	–	+	–	–	+	++	31	–	–	–	–
32	++	–	+	–	–	+	++	32	+	–	–	+
33	+	++	–	–	–	+	++	33	–	++	–	++
34	–	–	–	–	–	–	+	34	–	–	–	–
35	+	+	+	–	+	+	++	35	–	–	–	+
36	++++	–	–	–	–	–	+	36	++++	–	–	–
37	++	+	–	–	–	–	+	37	–	+	+++	++
38	–	–	+	–	–	–	–	38	–	–	–	+
39	+	+	–	–	–	–	+	39	–	–	–	++
40	++	+	–	–	–	+	+	40	–	+	+	++
41	++	+	–	–	–	–	+	41	+++	+	–	+
42	++	+	–	–	–	–	–	42	+++	–	–	–
43	–	–	–	–	–	–	–	43	–	–	–	–
44	–	–	–	–	–	–	–	44	–	–	–	–
45	++	–	–	–	–	–	–	45	+++	+	++	–
46	–	–	–	–	–	–	–	46	–	–	–	–
47	++	+	–	–	+	+	++	47	–	–	–	++
Bacterial								Bacterial				
48	+++	–	–	–	–	–	+	48	++++	++	+++	+
49	+++	–	–	–	–	–	–	49	++++	–	–	–
50	++++	–	–	–	–	++	++	50	++++	–	–	–
51	+	–	–	–	–	–	+	51	–	–	–	–
52	+++	–	+++	–	–	++	++	52	–	–	–	–
53	++++	–	–	–	–	+	–	53	++++	–	–	–
54	++++	–	+	–	–	–	+	54	++++	–	+++	++
55	+	–	–	–	–	+	+	55	–	+	–	+
56	++++	–	++++	–	+	++	+++	56	++++	++++	++	++++
57	++	+	–	–	–	+	++	57	++	–	–	+
58	++++	++	+++	–	–	++	+++	58	++++	+++	–	+
59	++	–	–	–	–	+	–	59	+	–	–	–
60	+++	+++	+	+	++	+	++	60	++++	++++	–	+
61	++	–	–	–	–	–	–	61	–	–	–	–
62	++	++	–	–	+	++	+	62	+	–	–	++
63	+	–	–	–	–	–	+	63	–	–	–	+
64	+	–	–	–	+	–	+	64	–	–	–	+++
65	+++	–	–	–	–	–	–	65	++++	–	–	–
66	+	+	–	–	+	–	+	66	–	–	–	–
67	–	–	–	–	+	–	–	67	–	–	+	–
68	–	+	–	–	+	–	+	68	–	–	–	+
69	++	+	–	–	+	+	++	69	++++	–	–	+
70	+	+	–	–	–	+	+	70	–	+	–	++
71	+++	+++	–	+	++	++	++	71	–	++++	–	+++
72	+++	+++	+	+	++	++	+++	72	–	++++	–	+++

[a] Peptide recognition was assessed as in Fig. 1. +, >10% specific lysis; ++, >20% specific lysis; +++, >40% specific lysis; ++++, >60% specific lysis.

Table III. *Continued*

Melan-A-Specific CTL Clones												
HD 421 2/7B12	HD 421 2/4G7	LAU 455/2	LAU 455/29	LAU 455/37	LAU 337/1B5	LAU 337/2A5	LAU 337/6C3	LAU 337/3A9	LAU 465/4E1	LAU 465/10B8	LAU 465/8D3	LAU 465/ 12A6
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	++	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	+++	−	−	+	−	−	−
−	−	−	−	−	−	+	−	−	−	−	−	−
−	−	−	−	−	−	+	−	−	−	−	−	−
−	−	−	−	−	−	+	−	−	−	−	−	−
−	++	−	−	−	+	++	−	−	+	−	−	−
+	+++	−	+++	+	+++	−	−	−	+	−	+	−
−	+	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	++	++	−	−	+	−	+	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	+	−	−	++	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	++	−	−	+	−	−	−
−	−	−	+	−	++	+++	−	−	++	−	−	−
−	−	−	−	−	−	+	−	−	+	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	+	−	−	−	−	−	−
++++	+++	++++	+++	+++	++++	++++	++++	++++	+++	+++	+	++
−	−	−	++	+	++	+++	−	+	+	−	−	−
−	−	−	+	−	+	+++	−	−	+	−	−	−
++	+++	+++	+++	+++	++++	++++	−	−	++	+	++	−
−	−	−	−	−	+	++	−	−	+	−	−	−
−	−	−	−	−	++	++	−	−	+	−	−	−
−	−	−	−	−	+	++	−	−	+	−	−	−
−	++	−	++	+	+++	+++	−	−	++	−	−	−
++	+++	−	−	−	++	+	−	−	−	−	+	+++
−	−	−	−	−	+	++	−	−	+	−	−	−
−	−	−	−	−	+	++	−	−	++	−	−	−
−	−	−	+	−	++	+++	−	−	++	−	−	−
−	−	−	−	−	+	++	−	−	+	−	−	−
−	−	−	−	−	++	++	−	−	+	−	−	−
−	−	−	−	−	++	++	−	−	+	−	−	−
−	++	−	+	−	++	++	−	−	++	−	−	−
−	−	−	−	−	+	++	−	−	−	−	−	−
−	−	−	−	−	++	++	−	−	+	−	−	−
−	−	−	−	−	+	++	−	−	+	−	−	−
−	−	−	−	−	++	+++	−	−	++	−	−	−
−	−	−	−	−	+	++	−	−	+	+	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	−	−	−	−	−	−	−
−	+	−	−	−	−	−	−	−	−	−	−	−
−	+	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	++	+++	−	−	+	−	−	−
−	−	−	−	−	−	+	−	−	−	−	−	−
−	−	−	−	−	+	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−
−	+	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	++	−	−	+	−	−	−
−	−	−	−	−	−	++	−	−	+	−	−	−
−	−	−	−	−	++	+++	−	−	+	−	−	−
−	++	+	++	++	+++	++++	−	−	++	−	−	−
−	+	+	++	+	+++	+++	−	−	++	−	−	−

Table IV. *Recognition of MSI 44 peptides by Melan-A-specific CTL*

Human	Lines[a]	Clones[a]	Viral	Lines	Clones	Bacterial	Lines	Clones
MSI 44-			MSI 44-			MSI 44-		
1	0	0	23	3	8	48	2	4
2	0	1	24	5	5	49	1	0
3	0	0	25	7	13	50	3	1
4	1	0	26	3	5	51	2	0
5	1	4	27	3	3	52	4	4
6	0	1	28	4	4	53	2	0
7	0	1	29	7	8	54	3	3
8	0	1	30	5	8	55	3	6
9	5[b]	5	31	4	3	56	5	6
10	6	9	32	4	4	57	4	3
11	4	1	33	4	6	58	5	6
12	6	5	34	1	3	59	2	0
13	1	0	35	6	4	60	7	7
14	3	2	36	2	3	61	1	0
15	0	1	37	3	8	62	5	4
16	0	0	38	1	3	63	2	2
17	2	2	39	3	5	64	3	3
18	6	6	40	4	6	65	1	1
19	2	2	41	3	5	66	4	0
20	1	0	42	2	4	67	1	2
21	4	2	43	0	0	68	3	4
			44	0	1	69	5	3
			45	1	3	70	4	5
			46	0	1	71	6	9
			47	5	4	72	7	9

[a] A total of 7 Melan-A-monospecific CTL lines and 16 Melan-A-specific CTL clones was tested.
[b] Numbers are underlined if representing at least 50% of the total CTL populations analyzed.

Table V. *Recognition of MSI 44 peptides by Melan-A-specific CTL*

CTL	Human	Viral	Bacterial	Total
Lines				
LAU 203	7[a]	18[b]	23[c]	48
LAU 181	4	12	10	26
HD 421	6	8	6	20
LAU 455	1	2	3	6
LAU 337	8	9	10	27
LAU 552	6	12	14	32
LAU 465	10	19	19	48
Clones				
LAU 203/17	3	11	8	22
LAU 203/1.3	1	5	4	10
HD 421/2/4A12	5	15	15	35
HD 421/2/7B12	1	2	1	4
HD 421/2/4G7	3	6	8	17
LAU 455/2	0	1	2	3
LAU 455/29	3	6	4	13
LAU 455/37	1	3	3	7
LAU 337/1B5	5	22	9	36
LAU 337/2A5	12	21	15	48
LAU 337/6C3	0	0	0	0
LAU 337/3A9	0	1	0	1
LAU 465/4E1	7	19	10	36
LAU 465/10B8	0	2	0	2
LAU 465/8D3	2	2	2	6
LAU 465/12A6	0	1	1	2

[a] Number of peptides recognized among 21 of self origin.
[b] Number of peptides recognized among 25 of viral origin.
[c] Number of peptides recognized among 25 of bacterial origin.

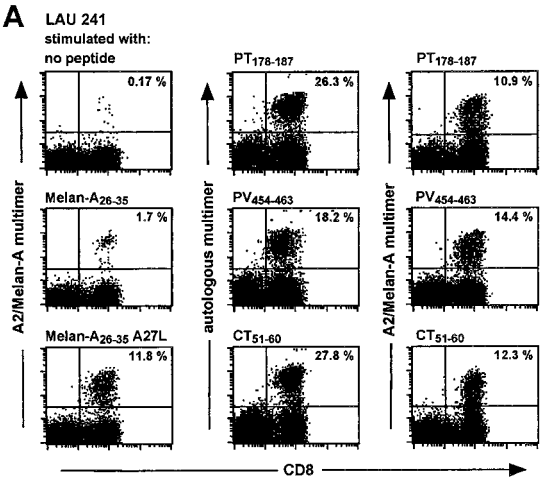
than $CD8^+$ Melan-A_{26-35} A27L multimer$^-$ fractions. In contrast, the peptide used for the in vitro stimulation was generally recognized by both fractions with roughly similar efficiency (Fig. 5*C*). Importantly, a low but significant level of specific lysis of Melan-A^+ $A2^+$ tumor cells was detected for the majority of the tested cultures and mostly segregated with the $CD8^+$ Melan-A_{26-35} A27L multimer$^+$ fractions (Fig. 5*D*).

Discussion

The identification of natural peptide sequences cross-recognized by clinically relevant T cell clones is of great interest for both the understanding of T cell repertoire selection and maintenance, as well as for immunotherapeutic purposes. In a review article of T cell cross-reactivity, Mason (6) estimated that a single T cell can productively interact with as many as 10^6 different MHC/peptide complexes. Despite this high degree of cross-reactivity and taking into account the large number of possible MHC-associated peptides (from 10^{10} for 9-mers to 10^{12} for 11-mers) the chances to identify a peptide cross-recognized by a given TCR among randomly selected peptides is theoretically less than one in 10^4. Therefore, it is necessary to design targeted methodologies that allow the rapid identification of such sequences. In this study, we tested the predictive potential of our recently described PS-SCL biometric data analysis (14) to use data previously generated by screening a PS-SCL with a Melan-A-specific CTL clone (16) to identify Melan-A cross-reactive sequences in a public protein database. It is of note that, in contrast to other approaches, no information on the parental sequence is required for this type of analysis, which could have been similarly performed for a clinically relevant T cell clone of unknown specificity. In addition, as this analytical approach is exclusively based on functional data and not on sequence homology between parental and cross-reactive peptides, it also allows the identification of cross-reactive peptides with totally unrelated sequences.

Validation of the high predictive value of our analytical approach came from the finding that a high proportion of the peptides analyzed, including sequences with the highest stimulatory scores of either self, viral, or bacterial origin, were recognized by the CTL clone used for the initial PS-SCL screening. It is noteworthy that recognition was highly specific as none of 100 unrelated peptides was significantly recognized (not shown). Reactive peptides were recognized with variable efficiency; remarkably, however, more than half of them were recognized with similar or higher efficiency as compared with the Melan-A parental decapeptide. Further support for the validity of our approach came from the correlation found between predicted stimulatory scores and peptide recognition. It is of note that the most active peptides were identified among peptides of bacterial origin, in good agreement with the higher predicted stimulatory score of this group. In addition, and interestingly, we also found a clear correlation between the relative predicted A2 binding efficiency of the retrieved peptides and their recognition. Thus, although a good A2 binding did not necessarily predict cross-recognition, omitting from the screening peptides with extremely poor predicted binding capacity would have further improved the likelihood of identifying peptides cross-recognized by the CTL clone used for the PS-SCL screening.

A surprising finding of this study was that a much higher proportion of peptides were cross-recognized by Melan-A-specific tumor-reactive CTL other than clone LAU 203/1.5 as compared with clone LAU 203/1.5 itself. However, the extent of cross-recognition was extremely variable among clones, ranging from no detectable cross-recognition (i.e., for clone LAU 337/6C3) to cross-recognition of 68% of the analyzed peptides (i.e., for clone LAU 337/2A5). Remarkably, these two clones recognized Melan-A peptides with comparable efficiency (24); thus, a difference in functional



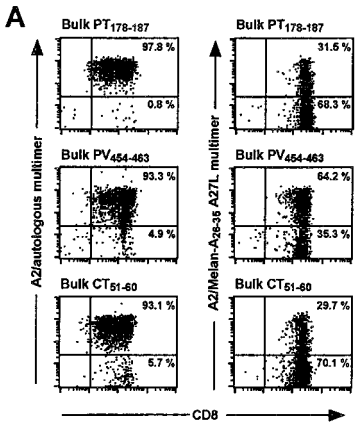
B

	Stimulated with:	Stained with: autologous multimers	Stained with: A2/Melan-A multimers
LAU 241	no peptide	na	0.17[a]
	Melan-A_{26-35}	na	1.7
	Melan-A_{26-35} A27L	na	11.8
	$PT_{178-187}$	26.3	10.9
	$PV_{454-463}$	18.2	14.4
	CT_{51-60}	27.8	12.3
LAU 203	no peptide	na	0.36
	Melan-A_{26-35}	na	1.7
	Melan-A_{26-35} A27L	na	9.0
	$PT_{178-187}$	2.9	1.4
	$PV_{454-463}$	6.3	4.9
	CT_{51-60}	17.5	7.8
LAU 97	no peptide	na	0.17
	Melan-A_{26-35}	na	6.3
	Melan-A_{26-35} A27L	na	20.6
	$PT_{178-187}$	26.3	8.3
	$PV_{454-463}$	18.8	16.1
	CT_{51-60}	33.3	11.0

a: percent multimer$^+$ in CD8$^+$ T cells
na = non applicable

FIGURE 4. In vitro immunogenicity of frequently recognized PS-SCL-retrieved peptides. Highly enriched CD8$^+$ T cells from three melanoma patients, LAU 97, LAU 203, and LAU 241, were stimulated with peptides Melan-A_{26-35}, Melan-A_{26-35} A27L, $PT_{178-187}$, $PV_{454-463}$, and CT_{51-60}. Cells were stained with A2/Melan-A multimers or with multimers incorporating the three retrieved peptides after two cycles of in vitro stimulation with the corresponding peptide. The results obtained for patient LAU 241 are shown in *A*, and those obtained for the three patients are summarized in *B*.

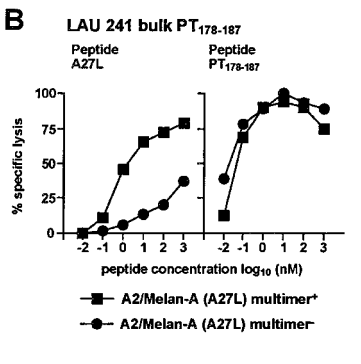
avidity of Ag recognition did not account for the different extent of cross-recognition, which could be due to differences in the fine specificity of Ag recognition of the clones analyzed. However, an alternative explanation could be that the different Melan-A-specific clones analyzed differ in terms of "degeneracy" of Ag recognition, e.g., in their capability to productively interact with a more-or-less large number of MHC/peptide complexes. The concept of "degeneracy" of Ag recognition (13) originates from the elucidation of the molecular interactions leading to T cell activation. It is based on the fact that, as underlined by the close inspection of crystal structures of TCR complexes with their MHC/peptide ligands (28, 29), most of the TCR contacts with the MHC/peptide complex are made with the MHC molecule rather than with the peptide. Thus, the majority of the TCR-MHC/peptide complex binding energy is determined by the affinity of a given TCR for the MHC molecule, the role of the peptide being only to modulate the latter and possibly to raise it beyond the threshold required for T cell activation. It follows that, if the TCR affinity for the MHC molecule is relatively low, the threshold can be attained with only few peptides and Ag recognition appears as highly selective. If, instead, the TCR affinity for the MHC is relatively high, activation is achieved by a relatively high number of different peptides and the TCR appears more "degenerate." Whether, and to what extent, peptides cross-recognized by the more degenerate TCR need to be structurally related is yet undetermined; however, it can be expected that at least a fraction of these peptides share some common structural and/or sequence features. In agreement with this hypothesis, while the strategy used to find Melan-A cross-reactive peptides was not conducted by searching for sequence homologies, the search appears to have yielded a group of cross-reactive peptides,



C

	Stimulated with:	Tested with: autologous peptide	Tested with: Melan-A_{26-35} A27L
LAU 241			
A2/Melan-A multimer$^+$	$PT_{178-187}$	0.05[a]	2
	$PV_{454-463}$	5	1
	CT_{51-60}	0.05	20
A2/Melan-A multimer$^-$	$PT_{178-187}$	0.01	> 1000
	$PV_{454-463}$	100	> 1000
	CT_{51-60}	0.01	1000
LAU 203			
A2/Melan-A multimer$^+$	$PT_{178-187}$	0.01	0.2
	$PV_{454-463}$	1	0.4
	CT_{51-60}	0.05	0.05
A2/Melan-A multimer$^-$	$PT_{178-187}$	0.02	> 1000
	$PV_{454-463}$	10	> 1000
	CT_{51-60}	< 0.01	> 1000

a:peptide dose (nM) required to achieve 50% maximal activity



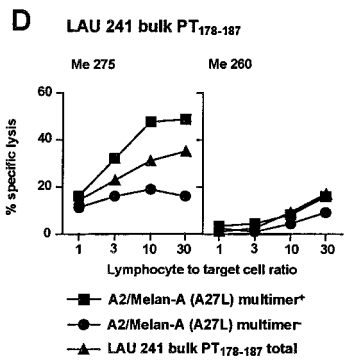


FIGURE 5. Heterogeneity of CTL responses elicited by Melan-A-related peptides. Autologous multimer$^+$ cell fractions (Fig. 4*A*) were isolated by multimer-guided cell sorting, expanded in vitro by PHA stimulation and stained again with autologous or A2/Melan-A_{26-35} A27L multimers and anti-CD8 mAb. Dot plots are shown for patient LAU 241 (*A*). A2/Melan-A_{26-35} A27L multimer$^+$ and multimer$^-$ fractions were further separated by cell sorting and their relative efficiency of peptide recognition was assessed in a CTL assay in the presence of graded peptide dilutions (*B*). Results obtained for all tested populations from patients LAU 203 and LAU 241 are summarized in *C*. The ability of A2/$PT_{178-187}$ multimer$^+$ cells from LAU 241, as well as of the corresponding A2/Melan-A_{26-35} (A27L) multimer$^+$ and multimer$^-$ fractions, to specifically lyse the A2$^+$ Melan-A$^+$ melanoma cell line Me 275 but not the A2$^-$ Melan-A$^+$ melanoma cell line Me 260 was assessed in a CTL assay in the absence of exogenously added peptide (*D*).

the majority of which share significant internal homology, particularly in the central part of the peptide (position 3 to 7). Thus, our search may have retrieved a "family" of Melan-A-related sequences, only part of which achieve the threshold of activation for clone LAU 203/1.5, whereas others achieve it only for more degenerate Melan-A-specific CTL.

Related to this is another important question on the modality of T cell cross-recognition. It has indeed not yet been established whether, and to what extent, T cell cross-recognition is focused or unfocused (6), i.e., whether or not distinct T cell clonotypes specific for the same Ag cross-recognize the same set of peptides. Whether cross-reactivity is focused or unfocused has important implications for the potential breaking of self-tolerance as the result of immune responses to foreign Ags. Indeed, if cross-reactivity is unfocused, the fortuitous cross-reaction between a foreign and a self peptide would lead to the expansion of a single or of a limited number of T cell clonotypes, whereas, in the opposite case, it could rapidly lead to a generalized autoimmune reaction. In this regard, the data obtained in this study support the latter hypothesis, as cross-reactivity was, in this case, at least partially focused. Indeed, although some peptides were recognized by only a few clonotypes, others were cross-recognized by the majority of Melan-A-specific CTL. The more frequently recognized peptides shared significant internal homology with Melan-A, particularly in the central part of the peptide. In contrast with the data obtained with the CTL clone used for the initial PS-SCL screening, there was no obvious correlation between the capacity of Melan-A-related sequences to be recognized by different Melan-A-specific CTL and the efficiency of their binding to A2. This indicates that cross-recognition of Melan-A-related peptides by different Melan-A-specific CTL is impacted more by the presence of TCR contact residues in the core region of the peptide than by their A2 binding efficiency. In addition, and importantly, in good agreement with recognition data, stimulation of PBMC from melanoma patients with the more frequently cross-recognized peptides elicited vigorous CTL responses that were at least partially cross-reactive with Melan-A.

It has been previously reported that Melan-A-like sequences are relatively frequent in proteins (30), possibly because the localization of this peptide in the transmembrane region of the Melan-A protein results in an amino acid composition that may be frequent in hydrophobic regions from other proteins. Thus, it could be argued that the high rate of cross-reactive sequences identified in this study is related to the high frequency of Melan-A-like sequences in proteins and that a very different result could be obtained for other epitopes. To address this point we have recently performed a similar analysis for an HLA-A2-restricted epitope from the cancer testis Ag SSX-2 (31). Interestingly, we found that the majority of retrieved sequences were cross-recognized by the CTL clone used for the PS-SCL screening (D. Valmori, manuscript in preparation), thus supporting the applicability of this approach to epitopes other than Melan-A.

What is the physiologic relevance of the Melan-A cross-reactive sequences? Melan-A cross-reactive peptides of self origin that are recognized by Melan-A-specific CTL with comparable or higher efficiency than Melan-A parental peptides are most likely not presented by thymic APC, as this would result in the deletion of Melan-A-specific thymocytes. In contrast, Melan-A-related self sequences that are recognized weakly or are able, when associated to an MHC molecule, to engage the TCR without leading to T cell activation (antagonists) may play a role in thymic positive selection of Melan-A-specific T cell precursors as well in the maintenance of the peripheral pool of Melan-A-specific T cells. Melan-A-related sequences of pathogen origin could be involved in the pathogenesis of vitiligo, a common progressive depigmentary condition of unknown etiology that is due to the autoimmune destruction of skin melanocytes. The spontaneous appearance of vitiligo has been associated with a favorable prognosis in melanoma patients (32, 33). Interestingly, higher frequencies of A2/Melan-A multimer$^+$ cells have been found in individuals with autoimmune vitiligo as compared with healthy controls (34). In this respect, it is noteworthy that our search retrieved 25 viral and 280 bacterial peptide sequences with higher predicted stimulatory values than Melan-A. Interestingly, some of these sequences were derived from common human pathogens and are thus of clear interest for investigating a potential pathogen-related etiology of vitiligo. Finally, it is of note that the Melan-A cross-reactive peptides identified in this study most likely represent only a small subgroup of the existing ones and are not necessarily among the most relevant. Depending on the likely rapid improvement of relevant technologies, it will be of great interest, in future studies, to extend this analysis to a large number of peptides, possibly selected from the ones that are naturally processed and presented, to unveil the most relevant cross-reactivity between Melan-A and the universe of peptides encoded by human and pathogen genomes.

In conclusion, the findings reported in this study strongly encourage the use of this methodology, which combines PS-SCL screening with T cell clones of interest and includes biometric analysis of the generated data, to further elucidate the molecular basis of T cell cross-reactivity. In addition, the reported data validate the predictive value of this approach for identifying in public protein databases the sequence of natural peptides cross-recognized by clinically relevant T cells.

Acknowledgments

We thank N. Montandon for excellent technical assistance and Dr. P. Batard for assistance in cell-sorting experiments.

References

1. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature 334:395.*
2. Ashton-Rickardt, P. G., L. Van Kaer, T. N. Schumacher, H. L. Ploegh, and S. Tonegawa. 1993. Peptide contributes to the specificity of positive selection of CD8$^+$ T cells in the thymus. *Cell 73:1041.*
3. Alam, S. M., P. J. Travers, J. L. Wung, W. Nasholds, S. Redpath, S. C. Jameson, and N. R. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature 381:616.*
4. Goldrath, A. W., and M. J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature 402:255.*
5. Dietrich, P. Y., P. R. Walker, A. L. Quiquerez, G. Perrin, V. Dutoit, D. Lienard, P. Guillaume, J. C. Cerottini, P. Romero, and D. Valmori. 2001. Melanoma patients respond to a cytotoxic T lymphocyte-defined self-peptide with diverse and nonoverlapping T-cell receptor repertoires. *Cancer Res. 61:2047.*
6. Mason, D. 1998. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol. Today 19:395.*
7. Baker, B. M., S. J. Gagnon, W. E. Biddison, and D. C. Wiley. 2000. Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling. *Immunity 13:475.*
8. Valmori, D., J. F. Fonteneau, C. M. Lizana, N. Gervois, D. Lienard, D. Rimoldi, V. Jongeneel, F. Jotereau, J. C. Cerottini, and P. Romero. 1998. Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. *J. Immunol. 160:1750.*
9. Madden, D. R., D. N. Garboczi, and D. C. Wiley. 1993. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell 75:693.*
10. Loftus, D. J., Y. Chen, D. G. Covell, V. H. Engelhard, and E. Appella. 1997. Differential contact of disparate class I/peptide complexes as the basis for epitope cross-recognition by a single T cell receptor. *J. Immunol. 158:3651.*
11. Hemmer, B., M. Vergelli, B. Gran, N. Ling, P. Conlon, C. Pinilla, R. Houghten, H. F. McFarland, and R. Martin. 1998. Predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *J. Immunol. 160:3631.*
12. Wilson, C. S., J. M. Moser, J. D. Altman, P. E. Jensen, and A. E. Lukacher. 1999. Cross-recognition of two middle T protein epitopes by immunodominant polyoma virus-specific CTL. *J. Immunol. 162:3933.*
13. Hemmer, B., M. Vergelli, C. Pinilla, R. Houghten, and R. Martin. 1998. Probing degeneracy in T-cell recognition using peptide combinatorial libraries. *Immunol. Today 19:163.*

14. Zhao, Y., B. Gran, C. Pinilla, S. Markovic-Plese, B. Hemmer, A. Tzou, L. W. Whitney, W. E. Biddison, R. Martin, and R. Simon. 2001. Combinatorial peptide libraries and biometric score matrices permit the quantitative analysis of specific and degenerate interactions between clonotypic TCR and MHC peptide ligands. *J. Immunol. 167:2130.*
15. Coulie, P. G., V. Brichard, A. Van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, J. P. Szikora, et al. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 180:35.*
16. Pinilla, C., V. Rubio-Godoy, V. Dutoit, P. Guillaume, R. Simon, Y. Zhao, R. A. Houghten, J. C. Cerottini, P. Romero, and D. Valmori. 2001. Combinatorial peptide libraries as an alternative approach to the identification of ligands for tumor-reactive cytolytic T lymphocytes. *Cancer Res. 61:5153.*
17. Valmori, D., N. Gervois, D. Rimoldi, J. F. Fonteneau, A. Bonelo, D. Lienard, L. Rivoltini, F. Jotereau, J. C. Cerottini, and P. Romero. 1998. Diversity of the fine specificity displayed by HLA-A*0201-restricted CTL specific for the immunodominant Melan-A/MART-1 antigenic peptide. [Published erratum appears in *1999 J. Immunol. 163:1093.*] *J. Immunol. 161:6956.*
18. Valmori, D., M. J. Pittet, D. Rimoldi, D. Lienard, R. Dunbar, V. Cerundolo, F. Lejeune, J. C. Cerottini, and P. Romero. 1999. An antigen-targeted approach to adoptive transfer therapy of cancer. *Cancer Res. 59:2167.*
19. Romero, P., P. R. Dunbar, D. Valmori, M. Pittet, G. S. Ogg, D. Rimoldi, J. L. Chen, D. Lienard, J. C. Cerottini, and V. Cerundolo. 1998. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J. Exp. Med. 188:1641.*
20. Rubio-Godoy, V., C. Pinilla, V. Dutoit, E. Borras, R. Simon, Y. Zhao, J. C. Cerottini, P. Romero, R. Houghten, and D. Valmori. 2002. Toward synthetic combinatorial peptide libraries in positional scanning format (PS-SCL)-based identification of $CD8^+$ tumor-reactive T-cell ligands: a comparative analysis of PS-SCL recognition by a single tumor-reactive $CD8^+$ cytolytic T-lymphocyte clone. *Cancer Res. 62:2058.*
21. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol. 152:163.*
22. Romero, P., N. Gervois, J. Schneider, P. Escobar, D. Valmori, C. Pannetier, A. Steinle, T. Wolfel, D. Lienard, V. Brichard, et al. 1997. Cytolytic T lymphocyte recognition of the immunodominant HLA-A*0201- restricted Melan-A/MART-1 antigenic peptide in melanoma. *J. Immunol. 159:2366.*
23. Valmori, D., V. Dutoit, D. Liénard, F. Lejeune, D. Speiser, D. Rimoldi, V. Cerundolo, P. Y. Dietrich, J.-C. Cerottini, and P. Romero. 2000. Tetramer-guided analysis of TCR β-chain usage reveals a large repertoire of Melan-A-specific $CD8^+$ T cells in melanoma patients. *J. Immunol. 165:533.*
24. Valmori, D., V. Dutoit, V. Schnuriger, A. L. Quiquerez, M. J. Pittet, P. Guillaume, V. Rubio-Godoy, P. R. Walker, D. Rimoldi, D. Lienard, et al. 2002. Vaccination with a Melan-A peptide selects an oligoclonal T cell population with increased functional avidity and tumor reactivity. *J. Immunol. 168: 4231.*
25. Dutoit, V., V. Rubio-Godoy, M. J. Pittet, A. Zippelius, P. Y. Dietrich, F. A. Legal, P. Guillaume, P. Romero, J. C. Cerottini, R. A. Houghten, et al. 2002. Degeneracy of antigen recognition as the molecular basis for the high frequency of naive A2/Melan-A peptide $multimer^+$ $CD8^+$ T cells in humans. *J. Exp. Med. 196:207.*
26. Pittet, M. J., D. Valmori, P. R. Dunbar, D. E. Speiser, D. Lienard, F. Lejeune, K. Fleischhauer, V. Cerundolo, J. C. Cerottini, and P. Romero. 1999. High frequencies of naive Melan-A/MART-1-specific $CD8^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J. Exp. Med. 190:705.*
27. Valmori, D., J. F. Fonteneau, S. Valitutti, N. Gervois, R. Dunbar, D. Lienard, D. Rimoldi, V. Cerundolo, F. Jotereau, J. C. Cerottini, et al. 1999. Optimal activation of tumor-reactive T cells by selected antigenic peptide analogues. *Int. Immunol. 11:1971.*
28. Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison, and D. C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature 384:134.*
29. Garcia, K. C., M. Degano, R. L. Stanfield, A. Brunmark, M. R. Jackson, P. A. Peterson, L. Teyton, and I. A. Wilson. 1996. An $\alpha\beta$ T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. *Science 274:209.*
30. Loftus, D. J., C. Castelli, T. M. Clay, P. Squarcina, F. M. Marincola, M. I. Nishimura, G. Parmiani, E. Appella, and L. Rivoltini. 1996. Identification of epitope mimics recognized by CTL reactive to the melanoma/melanocyte-derived peptide $MART\text{-}1_{27–35}$. *J. Exp. Med. 184:647.*
31. Rubio-Godoy, V., M. Ayyoub, V. Dutoit, C. Servis, A. Schink, D. Rimoldi, J. C. Cerottini, R. Simon, Y. Zhao, R. Houghten, et al. 2002. Combinatorial peptide library based identification of peptide ligands for tumor-reactive cytolytic T lymphocytes of unknown specificity. *Eur. J. Immunol. 32:2292.*
32. Nordlund, J. J., J. M. Kirkwood, B. M. Forget, G. Milton, D. M. Albert, and A. B. Lerner. 1983. Vitiligo in patients with metastatic melanoma: a good prognostic sign. *J. Am. Acad. Dermatol. 9:689.*
33. Duhra, P., and A. Ilchyshyn. 1991. Prolonged survival in metastatic malignant melanoma associated with vitiligo. *Clin. Exp. Dermatol. 16:303.*
34. Ogg, G. S., P. R. Dunbar, P. Romero, J. L. Chen, and V. Cerundolo. 1998. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J. Exp. Med. 188:1203.*