Specific Cytotoxic T Lymphocyte Responses Against Melan-A/MART1, Tyrosinase and Gp100 in Vitiligo by the Use of Major Histocompatibility Complex/Peptide Tetramers: the Role of Cellular Immunity in the Etiopathogenesis of Vitiligo

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Vitiligo is a common skin disease characterized by the presence of well circumscribed, depigmented, milky white macules devoid of identifiable melanocytes. Although the detection of circulating anti-melanocytic antibodies and of infiltrating lymphocytes at the margin of lesions supports the view that vitiligo is an autoimmune disorder, its etiology remains unknown. In particular, it is still a matter of debate whether the primary pathogenic role is exerted by humoral or cellular abnormal immune responses. In this study, the presence of specific cytotoxic T lymphocyte responses against the melanocyte differentiation antigens Melan-A/MART1, tyrosinase, and gp100 in vitiligo patients have been investigated by the use of major histocompatibility complex/peptide tetramers. High frequencies of circulating melanocyte-specific $CD8^+$ T cells were found in all vitiligo patients analyzed. These cells exerted anti-melanocytic cytotoxic activity *in vitro* and expressed skin-homing capacity. In one patient melanocyte-specific cells were characterized by an exceptionally high avidity for their peptide/major histocompatibility complex ligand. These findings strongly suggest a role for cellular immunity in the pathogenesis of vitiligo and impact on the common mechanisms of self tolerance. ***Key words: cytotoxic T lymphocytes/human/melanocyte differentiation antigen/vitiligo. J Invest Dermatol 117:326–332, 2001***

Vitiligo is a relatively frequent skin disease (1% of the general population) characterized by the presence of depigmented macules and patches as a consequence of local melanocyte loss. Although its etiology remains unknown, the detection of circulating anti-melanocytic antibodies (Naughton *et al*, 1983; Betterle *et al*, 1984; Song *et al*, 1994; Cui and Bystryn, 1995; Kemp *et al*, 1997; Merimsky *et al*, 1998; Okamoto *et al*, 1998) and of lymphocytic infiltration at the margin of lesions (al Badri *et al*, 1993b; Le Poole *et al*, 1996) in most patients with vitiligo strongly supports the view that vitiligo is an autoimmune disorder. In addition, the most effective treatments that induce repigmentation, such as psoralen and ultraviolet A or topical steroids (Pruvot-Sentous *et al*, 1992; Mosher *et al*, 1999), suppress immune reactivity (Mandel *et al*, 1997; Halder and Young, 2000), suggesting that their immunosuppressive mechanisms interfere with the mechanism that leads to damage of melanocytes (Bystryn, 1997; Mahmoud *et al*, 1998).

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Abbreviations: CLA, cutaneous lymphocyte-associated antigen; CTL, cytotoxic T lymphocyte; PBMC, peripheral blood mononuclear cells

The pathogenic role of melanocyte-specific autoantibodies in vitiligo is still a matter of debate. Although these antibodies have been shown to be able to damage pigment cells both *in vitro* (Cui *et al*, 1993) and *in vivo* (Gilhar *et al*, 1995), more recently it has been reported that specific autoantibodies for the melanocyte differentiation antigen tyrosinase are present at a low frequency in the sera of vitiligo patients (Kemp *et al*, 1997), and that such autoantibody titers may be found both in vitiligo-like depigmentation (melanoma-associated hypopigmentation) and in healthy controls (Merimsky *et al*, 1996). This suggests that other mechanisms may lead to melanocyte loss.

The patchy, rather than generalized, distribution of cutaneous depigmentation and the most frequent symmetrical distribution of lesions support the hypothesis that autoimmune melanocyte damage is induced by clones of lymphocytes with affinities for specific areas of skin, rather than by anti-melanocytic autoantibodies (al Badri *et al*, 1993a). Evidence for the involvement of cellular immunity in the etiopathogenesis of vitiligo is further provided by rare cases of inflammatory vitiligo (Le Poole *et al*, 1996) and by the interesting, although isolated, observation of frequent skin-homing melanocyte-specific cytotoxic T lymphocytes (CTL) in some vitiligo patients (Ogg *et al*, 1998).

Several melanocyte glycoproteins have been shown to be antigenic; namely, the melanocyte differentiation antigens Melan-A/MART1, the gp100, and the tyrosinase (Chen *et al*, 1995;

Marincola *et al*, 1996; Boon and Old, 1997; Kageshita *et al*, 1997; Van den Eynde and van der Bruggen, 1997). They are consistently expressed in normal melanocytes and are highly expressed in melanoma, where specific CTL-mediated immune reactivity has been demonstrated. It is therefore conceivable that immunodominant antigens are similar in melanoma and vitiligo and that a melanocyte-specific CTL response can play a part in the disappearance of epidermal melanocytes in vitiligo.

If peripheral autoreactive T cells specific for melanocyte-differentiation antigens exist, this implies an incomplete self tolerance achievement. Numerous studies now support the view that the fate of self specific T cells depends on the avidity of T cell receptor–self ligand interactions within the thymus (central tolerance) or in the periphery (peripheral tolerance) (Kappler *et al*, 1987; Kisielow *et al*, 1988; Guerder and Matzinger, 1992; Surh and Sprent, 1994). The processes of positive and negative selection are based on the stability of the interaction between T cell receptor and peptide/major histocompatibility complex. Positive selection occurs after short interactions (low avidity), whereas negative selection is thought to be a result of more prolonged interactions (high avidity) leading to activation-induced cell death (Williams *et al*, 1999; Kersh *et al*, 1998). Therefore, only antigen-specific T cells characterized by a low-avidity phenotype for self antigens are expected to remain unaffected by intrathymic and/or extrathymic deletion (Liu *et al*, 1995; Morgan *et al*, 1998; Lee *et al*, 1999; Wang *et al*, 1999).

In this study, we sought to investigate the presence of specific CTL against Melan-A/MART1, tyrosinase and gp100 antigens in vitiligo patients by the use of major histocompatibility complex/peptide tetramers and asked whether these cells could play a part in melanocytic destruction.

MATERIALS AND METHODS

Patients Fourteen patients with nonsegmental vitiligo (nine females and five males) were included in this study. The patients did not receive any treatment for at least 3 mo prior to examination. Surface area involvement was estimated according to the ''rule of nines''. Two patients had associated autoimmune disease (thyroiditis). Peripheral blood samples of 30 ml from each patient had been obtained after signed informed consent. Six HLA-A*0201-positive blood donors were used as controls.

HLA-DNA typing Molecular typing for HLA-A genes was performed by polymerase chain reaction-sequence-specific primers technique. High-resolution typing was performed in all the HLA-A*02 positive patients, with polymerase chain reaction–sequence-specific primers employing a specific set of primers dry solution (Olerup *et al*, 1993). Nine patients turned out to be HLA-A*0201-positive.

Synthetic peptides The following peptides were purchased from Neosystem (Strasbourg, France): A27L (an analog of the Melan-A/MART1$_{26–35}$ epitope carrying a substitution of Ala for Leu at position 2 from the NH2 terminus, ELAGIGILTV) (Valmori *et al*, 1998), gp100-209M (an analog of the G9$_{209}$ epitope carrying a substitution of Thr for Met at position 2, IMDQVPFSV) (Parkhurst *et al*, 1996) and tyrosinase$_{1–9}$ (MLLAVLYCL) (Van Wolfel *et al*, 1994). All peptides were >90% pure as indicated by analytical high-performance liquid chromatography. Lyophilized peptides were diluted in dimethyl sulfoxide and stored at −20°C.

Tetramers Production of major histocompatibility complex/peptide tetramers has been described in detail (Altman *et al*, 1996). Briefly, recombinant HLA-A2 heavy chain and β_2-microglobulin heterodimers were produced as inclusion bodies in *Escherichia coli* XA90F′LacQ1. After extensive washing, inclusion bodies were dissolved in 8 M urea and HLA monomeric proteins were refolded in the presence of 10–40 μg of the respective peptides by dilution in 100 mM Tris, 0.4 M Arginine, 2 mM ethylenediamine tetraacetic acid, pH 8.0, 0.5 mM oxidized glutathione, 5 mM reduced glutathione and protease inhibitors. Monomers were concentrated, dialyzed against Tris 10 mM, pH 8.0, and biotinylated with 6 μg per ml of BirA enzyme for 4 h at 30°C. Biotinylated complexes were dialyzed and purified by ion exchange chromatography (monoQ, Pharmacia France, St Quentin en Yvelines, France) to remove free biotin. Tetramerization was achieved by adding phycoerythrin (PE) -conjugated streptavidin (Immunotech, a Beckman-Coulter company, Marseille, France) at a 4:1 ratio, and controlled by gel filtration on a Superdex 200 (Pharmacia France).

Flow cytometry Cells were stained with PE-labeled tetramers (0.5 μg/10^6 cells) for 15 min at 37°C; after two washes, indirect double-staining was performed using anti-human CD8 monoclonal antibody (OKT8, IgG2a) and fluorescein isothiocyanate (FITC) -labeled goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as second antibody. The specificity of each tetramer was confirmed by staining CTL lines or clones specific for HLA–A2 in association with the peptide of interest (not shown). To minimize background staining, tetramers were titrated and used at the lowest concentration that still gave a discernible population. A stringent gate for tetramer$^+$ cells, which did not include tetramer^{low} cells, was always set to avoid overestimation of the positive cells. The limit of detection of each tetramer had been previously determined to be 0.04% of CD8$^+$ T cells for A2/Melan-A and A2/tyrosinase, and 0.06% of CD8$^+$ T cells for A2/gp100 (Palermo *et al*, 2001). Triple staining with A2/Melan-A tetramer^{PE}, anti-CD45RA^{CYC} (IgG2b; Becton-Dickinson, Mountain View, CA) and either anti-CD45RO^{FITC} (IgG2a; Caltag Laboratories, Burlingame, CA) or anti-CD56^{FITC} (IgG2a; Southern Biotechnology Associates), were performed to assess the naive/memory phenotype of tetramer$^+$ cells. FITC-labeled anti-cutaneous lymphocyte-associated antigen (anti-CLA) monoclonal antibody HECA-452 (rat IgM; Becton-Dickinson) was used to stain CTL clones for 30 min on ice. Cells were analyzed on a FACScalibur (Becton-Dickinson). Data were analyzed using the CellQuest software. Lymphocytes were gated by forward and side scatter profile. Dead cells were excluded by propidium iodide staining.

T cell lines and clones For the generation of peptide-specific T cell lines, peripheral blood mononuclear cells (PBMC) from patients were isolated by Ficoll centrifugation (Ficoll-Paque; Amersham Pharmacia Biotech, Uppsala, Sweden) and monocyte-depleted by exclusion of plastic adherent cells. We cultured 10^5 per well PBMC in complete RPMI medium supplemented with 5% human serum, in the presence of 10^5 peptide-pulsed T2 cells. T2 cells are HLA-A*0201 human lymphoid cells that are defective in antigen processing but effectively present exogenously supplied peptides (Ljunggren *et al*, 1990). T2 loading was done in serum-free medium (X-VIVO 15, BioWhittaker, Walkersville, MD) using 2 μg per ml of A27L and 3 μg per ml of gp100-209M peptides following overnight incubation at 37°C. Low-dose (25 U per ml) interleukin-2 was added on day 5. For the cloning experiments, cells were plated at 0.3 cells per well in complete RPMI medium in the presence of irradiated PBMC (5×10^5 cells per ml), 2 μg per ml phytohemagglutinin and 250 U per ml recombinant interleukin-2. Proliferating clones were expanded in complete RPMI medium supplemented with 5% human serum and 500 U per ml recombinant interleukin-2.

Cytotoxicity analysis Peptide-specific T cell lines and clones were assayed for specific lysis using either peptide-pulsed T2 cells or two HLA-matched melanoma cell lines (501 mel and 213 mel, kind gifts of L. Rivoltini, Cancer Immunotherapy Unit, Istituto Nazionale Tumori, Milano) in a standard 4 h chromium release assay. Briefly, 10^6 target cells were labeled with 200 μCi ^{51}Cr for either 1.5 h (melanoma cells) or 15 h (T2 cells). T2 cells were pulsed with 3 μg per ml Melan-A/MART1 peptides for 30 min at room temperature. T2 cells pulsed with a HLA-A*0201 restricted, irrelevant peptide (gp100-209M) were used in each assay as a negative control at the same concentration as the relevant peptides. We plated 10^3 target cells per well and effector cells were added to give final E/T ratios ranging between 50 : 1 and 3 : 1. After 4 h at 37°C, 25 μl supernatant was collected, seeded in Lumaplate 96 solid scintillation plates (Packard Instruments Company, Meriden, CT) and counted in a β counter. The percentage of specific lysis was calculated as: $100 \times$ (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was assessed by incubating target cells in the absence of effectors and maximum release was determined in the presence of 1% Nonidet P-40 detergent (BDH Biochemicals, Poole, U.K.).

Avidity Titration curves over a wide range of peptide concentrations (1.2×10^{-8} M to 1.5×10^{-12} M) were generated and the avidity of the different clones was determined as the concentration of peptide required to obtain half maximal lysis ([nM] 50%) of target cells in standard cytotoxicity tests at a low E/T cell ratio (5 : 1).

Table I. Clinical and immunogenetical findings of the patients

Patient	Sex[a]	Duration (y)	Course[b]	Disease extension (%)	Associated diseases	HLA-A alleles
1 RSA	M	21	Stable	Hands, penis (13%)	Hypertension, NIDDM[c]	0201
2 PSA	F	20	Stable	Diffuse (80%)	No	0201
3 TSC	F	25	Stable	Abdomen, feet (18%)	No	0201,11
4 SRI	M	10	Progression	Diffuse (80%)	Psoriasis	0201,11
5 CLA	F	8	Stable	Arms (18%)	Hypertension	0201,68
6 ADL	M	40	Stable	Diffuse (80%)	Glaucoma	0201,31012
7 RNA	F	21	Stable	Diffuse (80%)	No	0201,2301
8 LPA	M	10	Stable	Face, arms, legs (45%)	No	0201,25
9 MBO	F	2	Progression	Hands, face (18%)	Thyroiditis, MsAb[d]	0201,30
10 RGI	M	0.5	Progression	Face, right arm (18%)	No	26,30
11 MRO	F	11	Stable	Hands (9%)	No	01,2301
12 IMO	F	8	Slow progression	Hands (9%)	Thyroiditis, MsAb[d]	3301,6601
13 ASC	F	14	Stable	Hands (9%)	No	03,30
14 RCA	F	11	Stable	Diffuse (80%)	No	0301,26

[a]M, male; F, female.
[b]The disease course was defined as active vitiligo (newly depigmented lesions during the last three months/progression or slow progression) or stable vitiligo (no recent depigmented or repigmented lesions in the three months prior to examination).
[c]NIDDM, non insulin-dependent-diabetes mellitus.
[d]MsAb, thyroid microsomal antibodies.

Statistical analysis The Mann–Whitney test was used to compare differences in the percentages of tetramer$^+$ cells found in vitiligo patients *vs* healthy donors.

RESULTS

High frequencies of circulating melanocyte-specific CD8$^+$ T lymphocytes in vitiligo patients To assess whether melanocyte-specific CD8$^+$ T lymphocytes are commonly present in vitiligo patients, we synthesized three fluorescent HLA-A2 tetramers complexed with peptides derived from melanocyte differentiation antigens: A2/Melan-A, A2/tyrosinase, and A2/gp100. We used these three tetramers to stain PBMC from 14 vitiligo patients directly *ex vivo*, without any antigen-specific stimulation. Clinical and immunogenetic characteristics of the patients are presented in **Table I**. High numbers of both Melan-A/MART1- and tyrosinase-specific cells were detected in all nine HLA-A*0201 patients (**Table II**). Detection was dependent on the expression of HLA-A*0201, as none of five A*0201-negative vitiligo controls had detectable tetramer$^+$ cells *ex vivo* (not shown). The frequency of A2/Melan-A tetramer$^+$ cells varied between 0.07% and 0.28% (mean 0.14%) of the total CD8$^+$ population, and the frequency of A2/tyrosinase-positive cells between 0.10% and 0.22% (mean 0.16%) (**Table II and Fig 1**). On the contrary, no gp100-specific CD8$^+$ T cells could be observed directly *ex vivo* by tetramer staining (**Table II**). This could mean either that CD8$^+$ cells specific for the peptide were not present or that their frequency was below the detection limit of tetramer staining. To discriminate between these two possibilities, PBMC from four selected patients were stimulated at 3 wk intervals using antigen-presenting cells (T2 cells) loaded with gp100-209M peptide. On day 8 after each stimulation cycle, recovered cells were monitored for the presence of CD8$^+$/tetramer$^+$ cells. After three stimulation cycles a significant enrichment of tetramer$^+$ cells was obtained in two of 11 cell lines established from patient PSA (from 0.04% to 4.3% tetramer$^+$ cells) (**Fig 2**), but from none of 11 lines established from patient MBO, six lines established from patient LPA and three lines established from patient TSC (not shown). These findings support the occasional presence of gp100-specific CTL in vitiligo patients, but are indicative of a low precursor frequency of these cells.

In the light of the recent evidence that Melan-A/MART1-specific CD8$^+$ cells exist also in some HLA-A*0201 normal individuals (Pittet *et al*, 1999), PBMC from six A*0201-positive normal donors were analyzed with the A2/Melan-A tetramer. In two cases we could detect discrete populations of tetramer$^+$ cells, which represented 0.07 and 0.13% of the total CD8$^+$ T cell population. Whereas naive T cells represented a variable proportion of the patients' tetramer$^+$ lymphocytes (between 36% and 60%, mean 47.4%, data not shown), virtually all the Melan-A-specific cells found in these two healthy donors displayed a naive phenotype (80% and 90% were CD56$^-$ CD45RA^{high} CD45RO$^-$, data not shown), in agreement with previous data (Pittet *et al*, 1999; Dunbar *et al*, 2000). In all the other healthy donors tested tetramer$^+$ cells were detected at frequencies close to or below our estimated detection limit for A2/Melan-A tetramer staining (**Table II, Fig 1**). Overall, the frequency of tetramer$^+$ cells found in the peripheral blood of healthy controls appeared to be significantly lower than that found in vitiligo patients ($p = 0.015$). In view, however, of the smallness of the compared populations (nine patients *vs* six healthy donors) we consider this statistical result a strong indication, yet not a formal demonstration, of the difference between these two groups.

Table II.Percentages of circulating TAA-specific CTLs in HLA-A*0201 vitiligo patients and healthy donors

	Vitiligo patients			Healthy donors	
	A2-tetramer$^+$ cells[a]			A2-tetramer$^+$ cells[a]	
Patient	Melan-A	Tyrosinase	gp100	Donor	Melan-A
1 RSA	0.07	0.10	0.01[b]	15 GFI	0.13
2 PSA	0.19	0.20	0.04[b]	16 RBI	0.01[b]
3 TSC	0.22	0.22	0.00[b]	17 MSO	0.07
4 SRI	0.09	0.12	–	18 VPU	0.03[b]
5 CLA	0.07	0.14	0.04[b]	19 RMA	0.00[b]
6 ADL	0.07	0.14	0.02[b]	20 MES	0.02[b]
7 RNA	0.09	0.13	0.06[b]		
8 LPA	0.21	0.19	0.01[b]		
9 MBO	0.28	0.21	0.04[b]		

[a]Percentage of tetramer$^+$ cells relative to total CD8$^+$ T cells.
[b]Percentage values below the limit of detection.

Anti-melanocytic cytotoxic activity of tetramer$^+$ cells from vitiligo patients We established several Melan-A/MART1-specific cell lines from three patients and two donors through repeated PBMC antigen-specific stimulation *in vitro*. Ten of 11 cell

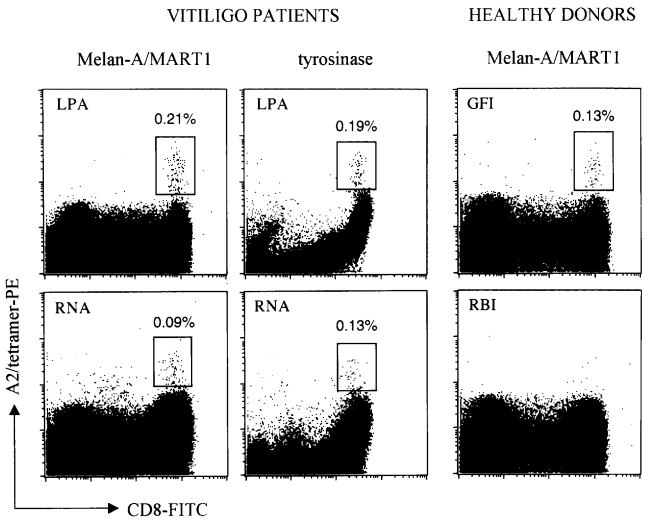

Figure 1. Enumeration of melanocyte-specific CD8$^+$ T cells by tetramer staining. Cells were gated by forward- and side-scatter for lymphocytes. Gated populations are plotted as CD8-staining (horizontal axis) *vs* tetramer-staining (vertical axis). Each column shows the staining for a different peptide-loaded HLA-A*0201 tetramer. Boxed populations, CD8$^+$/tetramer$^+$ cells (frequencies within total CD8$^+$ T cells above each box). Upper left corner, patient/donor identifiers.

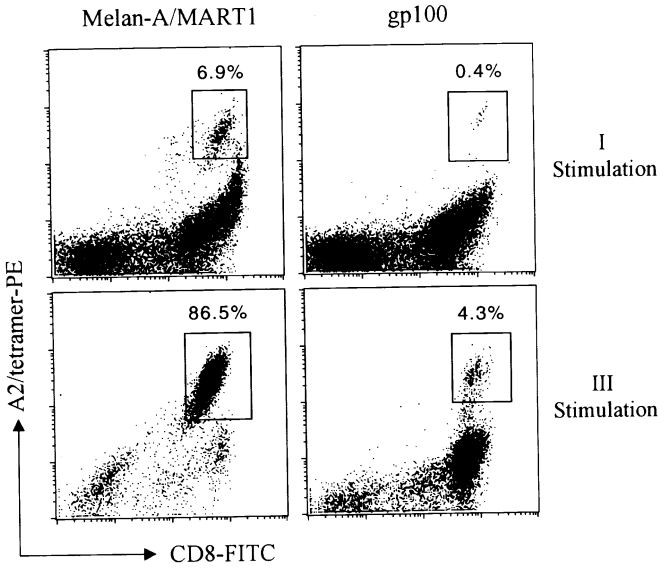

Figure 2. Direct visualization of *in vitro* expansion of Melan-A/MART1- and gp100-specific precursors from one vitiligo patient. Total PBMC from patient PSA were stimulated at 3 wk intervals with peptide-pulsed, irradiated T2 cells as antigen-presenting cells. Peptides used for pulsing were either A27L or gp100-209M. Cultures were stained on day 8 after each stimulation with tetramers^{PE} and anti-CD8^{FITC} monoclonal antibody. Boxed populations, CD8$^+$/tetramer$^+$ cells (frequencies within total CD8$^+$ T cells above each box). On the right side, number of stimulation cycles.

lines from patient PSA, five of eight lines from patient LPA and six of 13 lines from patient MBO could be successfully expanded up to 5×10^6 cells, thus demonstrating a consistent ability of these cells to proliferate in culture (see **Fig 2** for one representative case). At the end of the culture, cell lines contained from 12- to 470-fold enriched tetramer$^+$ cells compared with the frequency calculated in PBMC. We used these lines to assess whether the frequency of tetramer$^+$ T cells detected in bulk cultures correlated with the peptide-specific cytotoxicity measured by chromium release assay. We found that the increasing percentage of tetramer$^+$ cells in the different cultures directly correlated with the specific cytotoxic activity against target cells pulsed with the Melan-A/MART1A27L peptide (**Fig 3**), thus confirming the antigen-specificity of tetramer$^+$ cells. On the contrary, the tetramer$^+$ cells found in the two healthy donors possessed a reduced *in vitro* expansion potential following antigen-specific stimulation, as was demonstrated by our inability to obtain a comparable enrichment of tetramer$^+$ cells (not shown). Importantly, none of the cell lines established from these healthy donors exhibited lytic activity against target cells pulsed with the Melan-A/MART1A27L peptide (data not shown).

Next, we examined whether the melanocyte-specific CTL could be capable of lysing HLA-matched melanocyte cell lines from melanoma patients. T cell clones were generated by limiting dilution from a Melan-A/MART1-sensitized cell line from patient PSA. Their ability to lyze both peptide-pulsed target cells and two different HLA-A*0201-positive melanoma cell lines, which present the peptide through endogenous processing pathways, was tested. We found that all CD8$^+$ A2/Melan-A tetramer$^+$ clones were able to lyze peptide-pulsed targets and five of these exerted a very efficient lytic activity against melanoma cells (**Fig 4**).

Finally, we examined the skin-homing capacity of Melan-A/MART1-specific CTL clones by analyzing surface expression of the skin-homing receptor, CLA. Although lower CLA staining levels were reported for lymphocytes kept in culture than for uncultured cells (Fuhlbrigge *et al*, 1997), we found that half of the clones analyzed (PSA.4.1, PSA.4.7, PSA.4.8, PSA.4.12) expressed high levels of CLA, about 500-fold over background (data not shown).

High avidity of melanocyte-specific CTL Eight Melan-A/MART1-specific CTL clones established from patient PSA were selected and their relative avidity measured. Interestingly, the relative avidity of all clones turned out to be extremely high, with [nM] 50% comprised between 0.3 and 11 (**Fig 5**). We found that clone avidity directly correlated with *in vitro* anti-melanocytic activity of CTL, as was demonstrated by the very efficient lysis of melanoma cells we obtained with the five highest avidity clones (PSA.4.8, PSA.4.7, PSA.4.1, PSA.4.12, PSA.4.33; **Figs 4 and 5**). The peptide-specific, nontumor-reactive, clones PSA.4.27, PSA.4.16, and PSA.4.9 exhibited a lower avidity than the first five (**Fig 5**).

DISCUSSION

This study demonstrates the presence of high frequencies of melanocyte-specific CTL in the peripheral blood of vitiligo patients. These autoreactive CTL exhibited intact functional properties, as demonstrated by their consistent ability to undergo *in vitro* expansion and to lyze peptide-pulsed antigen-presenting cells. Notably, they were also capable of lysing HLA-matched melanoma cell lines more efficiently than CTL obtained from melanoma patients (Palermo *et al*, 2001). This last feature is particularly interesting because of the impact that the demonstration of a superior anti-tumor activity of vitiligo cells might have on melanoma immunotherapeutical intervention. Lysis of cultured melanocytes, however, which would be more relevant for vitiligo pathogenesis, remains to be demonstrated. A proportion of these anti-melanocyte CTL expressed the skin-homing receptor, cutaneous lymphocyte antigen CLA, suggesting that circulating melanocyte-specific autoreactive T lymphocytes in vitiligo patients can be rapidly addressed to the skin. Overall, these findings show a clear association between the presence of melanocyte-specific CTL and vitiligo, suggesting a pathogenic role for antigen-specific T cells in this disease.

Although we documented a significantly lower frequency of Melan-A/MART1-specific CTL in healthy donors than in vitiligo patients ($p = 0.015$), the occasional existence of CTL precursors to

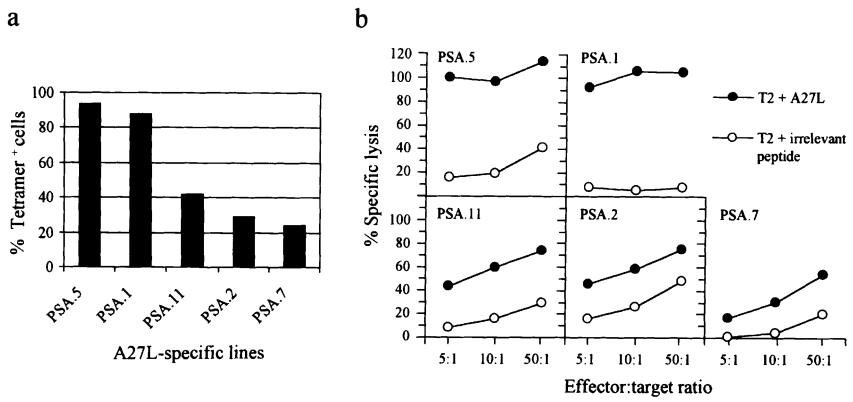

Figure 3. Correlation between the frequency of tetramer$^+$ T cells detected in bulk cultures and the peptide-specific cytotoxicity. (*a*) Five A27L-specific T cell lines established from patient PSA through peptide-specific stimulation were stained with the A2/Melan-A tetramer. Percentages of tetramer$^+$ cells in the different lines are shown as histograms. (*b*) The five A27L-specific T cell lines were tested in standard 4 h chromium release assays for their ability to lyze HLA-A*0201 T2 cells pulsed with A27L peptide, at different E/T cell ratios. Lysis of T2 cells pulsed with an irrelevant peptide is shown as the control. Points are means of triplicate. Shown is one of three experiments with similar results.

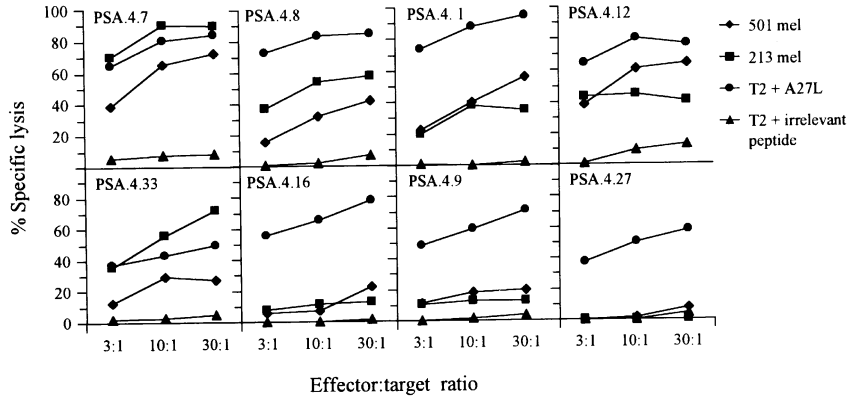

Figure 4. Tumor-reactivity of Melan-A/MART1-specific CTL clones. Eight T cell clones, derived by limiting dilution from one Melan-A/MART1-sensitized cell line of patient PSA, were tested in standard 4 h chromium release assays for their ability to lyze T2 cells pulsed with A27L peptide as well as two different, HLA-matched, melanoma cell lines (501 mel and 213 mel). Lysis of T2 cells pulsed with an irrelevant peptide is shown as the control. Points are means of triplicate. Shown is one of several experiments with similar results.

this self antigen in healthy blood donors is not fully understood. They might represent CTL specific for environmentally encountered antigens cross-reacting with human Melan-A/MART1 (Loftus *et al*, 1996).

An unusual facet of vitiligo is its relation to melanoma. CTL directed to self antigens shared by normal melanocytes and melanoma cells are found in both conditions and imply a breakdown of tolerance, yet the resulting immune reaction is the opposite. In this respect, reactivity to vitiligo melanocytes may be regarded as the effective variant of an immune response often ineffective in melanoma. The mechanisms at the basis of these opposite effects are not known. An interesting, although controversial, possibility is that vitiligo melanocytes have intrinsic differences from normal melanocytes with respect to the expression of immune-related molecules that could explain abnormal immune response in vitiligo (al Badri *et al*, 1993a; Hedley *et al*, 1998). Upregulation of these surface molecules could enhance antigenicity of vitiligo melanocytes and elicit an autoimmune response. On the other hand, this study demonstrates an extremely high avidity in antigen recognition by the anti-melanocyte CTL clones derived from one vitiligo patient, as demonstrated by the very low major histocompatibility complex/peptide densities required to trigger cytolysis in all eight Melan-A/MART1-specific clones analyzed ([nM] 50% between 0.3 and 11). [nM] 50% values reported thus far for CTL clones, whose reactivity to A*0201-presented, melanoma-derived self peptides have been documented, widely range from 0.2 to 500 nM (Appella *et al*, 1995; Valmori *et al*, 1999a, b). Melan-A/MART1-specific vitiligo clones appeared on the contrary to be selected for a very narrow range of high avidities. We suggest that high avidity in antigen recognition might represent a peculiar property of anti-melanocytic CTL from vitiligo patients that contribute to their abnormal reactivity. The strong humoral response described for vitiligo patients might be an important secondary phenomenon contributing to the fact that autoimmunity is effective in vitiligo, whereas in melanoma it is not.

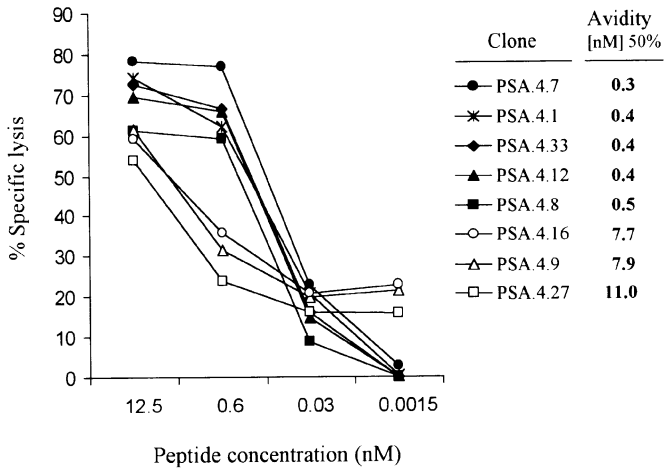

Figure 5. Avidity of Melan-A/MART1-reactive CD8$^+$ T cell clones. Eight T cell clones, derived by limiting dilution from Melan-A/MART1-sensitized cell lines of patient PSA, were tested in standard 4 h chromium release assays for their ability to lyze HLA-A*0201 T2 cells pulsed with decreasing concentrations of Melan-A/MART1 A27L peptide, at an E/T cell ratio of 5 : 1. Avidity ([nM] 50%) was defined as the concentration of peptide required to obtain half maximal lysis of target cells. At the same E/T cell ratio, lyses of empty T2 cells used as controls were as follows: PSA.4.7, 3.7%; PSA.4.1, 0.6%; PSA.4.33, 0.9%; PSA.4.12, 0.4%; PSA.4.8, 0.7%; PSA.4.16, 18%; PSA.4.9, 19%; PSA.4.27, 15%. Points are means of triplicate. Shown is one of three experiments with similar results.

The presence of anti-melanocytic CTL exhibiting such a high avidity was somehow unexpected in terms of self tolerance. In fact, the most effective way to achieve tolerance to self antigens is

thymic deletion, by which thymocytes recognizing self peptides with high avidity undergo apoptosis (Kappler *et al*, 1987; Kisielow *et al*, 1988; Surh and Sprent, 1994). Only T cells that recognize self epitopes with an avidity below a certain threshold are permitted access to the periphery (Liu *et al*, 1995; Lee *et al*, 1999). Accordingly, it was demonstrated that tolerance to the self antigen p53 was achieved by selectively eliminating T cells with high avidity (Hernandez *et al*, 2000). Vitiligo CTL must have eluded this central tolerance mechanism. In the periphery, tolerance may be achieved by a variety of mechanisms, including clonal deletion, anergy and ignorance. Here, T helper cells become fundamental to tolerize a wide variety of potentially autoimmune CTL. If peripheral T help is available, CTL precursors that recognize antigens are activated, while in the absence of help, they are tolerized (Guerder and Matzinger, 1992). In vitiligo, however, the high titers of anti-melanocytic IgG antibodies strongly indicate the presence of specific T helper responses.

The definition of the mechanisms by which tolerance is established to an antigen and those by which tolerance can be terminated are crucial for understanding the generation of autoimmune responses. The presence of frequent, high avidity autoreactive CTL in vitiligo suggests a role for cellular immunity in the etiology of this disease, but the origin of these cells remains elusive in the light of the current models of self tolerance. We will test in the near future the possibility that these autoreactive CTL are resistant to activation-induced cell death.

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