HLA-A2 Restricted, Melanocyte-Specific CD8$^+$ T Lymphocytes Detected in Vitiligo Patients are Related to Disease Activity and are Predominantly Directed Against MelanA/MART1

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Vitiligo is a skin and hair disorder characterized by circumscribed depigmented lesions due to lack of melanocytes in the respective areas. It has been suggested that vitiligo is caused by an autoimmune-mediated destruction of melanocytes. Recently, the presence of a high frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in the peripheral blood of patients with vitiligo was reported. Our study examines the frequency of melanocyte-specific cytotoxic T lymphocytes in vitiligo patients and its relationship to disease activity. Thirty-two patients with moderate to active vitiligo and 17 control subjects were included. Melanocyte specific reactive CD8$^+$ T cells were identified by enzyme-linked immunospot assay after stimulation with five peptides from gp100, four peptides from MelanA/MART1, and two peptides from tyrosinase. In selected patients, intracellular interferon-γ staining for the detection of specific reactive CD8$^+$ T cells was additionally performed. In seven of 10 patients (70%) with actively progressive disease CD8$^+$ T cells directed against melanocyte epitopes were detected, whereas only in four of 22 patients (18%) with moderate disease activity such specific reactivity was found. MelanA/MART1 peptides were immunodominant in nine patients reacting against EAAGIGILTV and three patients reacting against ILTVILGVL. Intracellular interferon-γ staining confirmed the findings obtained by the enzyme-linked immunospot technique. The present study supports the hypothesis that vitiligo is a cytotoxic T lymphocyte-mediated autoimmune disease. The presence of melanocyte-specific reactive CD8$^+$ T cells seems to be closely related to disease activity. ***Key words: ELISPOT/gp100/intracellular IFN-γ staining/tyrosinase. J Invest Dermatol 116:891–897, 2001***

Vitiligo is a skin and hair disorder characterized by circumscribed depigmented lesions of variable size and shape, which tend to enlarge over time and in some cases cover the whole body. This disease was found at a prevalence of 0.4–1% of the world's population, regardless of sex, age, and color of the skin. In 30–40% there is a positive family history. The etiology of vitiligo is not yet clearly known (Chakraborty *et al*, 1996; Maresca *et al*, 1997), but it is believed by many authors that vitiligo is caused by an autoimmune-mediated destruction of melanocytes in the epidermis. It has been suggested that this destruction is caused by autoantibodies (Naughton *et al*, 1983; Norris *et al*, 1988; Baharav *et al*, 1996; Hann *et al*, 1996; Fishman *et al*, 1997) or may be mediated by cellular immunity (Le *et al*, 1996; Mahmoud *et al*, 1998).

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Abbreviations: CD, cluster of differentiation; CLA, cutaneous lymphocyte antigen; CMV, cytomegalovirus; CTL, cytotoxic T lymphocytes; ELISPOT, enzyme-linked immunospot; PBMC, peripheral blood mononuclear cells; PBS/T, PBS with 0.05% Tween 20.

It is an interesting finding that vitiligo was found more frequently in melanoma patients (Nordlund *et al*, 1983; Duhra and Ilchyshyn, 1991; Cui and Bystryn, 1995; Cavallari *et al*, 1996). The prevalence of vitiligo in patients with metastatic melanoma is 2–4%, which is clearly higher than in the normal population. Furthermore, vitiligo was observed following successful immunotherapy of melanoma (Lacour *et al*, 1992; van Elsas *et al*, 1999; Overwijk *et al*, 1999). Particularly after immunization of melanoma patients with peptide pulsed dendritic cells, triggering tumor peptide-specific cytotoxic T lymphocyte (CTL) response, and likewise, following high-dose interleukin-2 therapy, vitiligo-like depigmentations were documented (Rosenberg and White, 1996; Nestle *et al*, 1998). Furthermore, the presence of clonally expanded T cells with identical BV regions has been observed in areas of melanocyte destruction of both vitiligo-like leukoderma and melanoma regression (Becker *et al*, 1999). Recently, Ogg *et al* (1998) demonstrated the presence of a high frequency of skin-homing melanocyte-specific CTL in the peripheral blood of seven of nine patients with vitiligo.

This study was planned in order to correlate the occurrence of such T cells with disease state. We established a sensitive enzyme-linked immunospot (ELISPOT) assay for detection of CD8$^+$ T cells specific for 11 different peptides presented by melanocytes from MelanA/MART1, tyrosinase and gp100. Furthermore, we applied

intracellular interferon (IFN) -γ staining as an additional marker for antigen specificity, and we examined the expression of the skin homing marker cutaneous lymphocyte antigen (CLA) on these cells.

MATERIALS AND METHODS

Study subjects Thirty-two patients with active to moderately progressive vitiligo and 17 healthy asymptotic persons as control individuals were included in this study after obtaining informed consent. All of these study subjects were HLA-A*0201-positive.

The disease classification of vitiligo was based on case history. Patients with 10% and higher increase of vitiligo areas during the last 3 mo were classified as actively progressive, patients with less increase but signs of progression for the last 6 mo were classified as moderately progressive, and patients without a clear increase of vitiligo areas during the last 6 mo were classified as having stable disease. Altogether, 137 patients with vitiligo were screened (76 with stable disease, 61 with active to moderately progressive disease; 32 of 61 were HLA-A*0201 positive).

Ten of the 32 vitiligo patients included were classified as having actively progressive disease and 22 patients as having moderately progressive disease. Three of the 10 patients with actively progressive disease had a vitiligo associated autoimmune disease, two thyroid disease and one pernicious anemia.

The 17 control individuals were not known to have familiar disposition of any autoimmune disorder.

Lymphocyte preparation Peripheral blood mononuclear cells (PBMC) were isolated from 100 ml heparinized blood samples by density gradient centrifugation using Ficoll (FicoLite Linearis, Bettingen/Main, Germany). PBMC were washed four times with phosphate-buffered saline (PBS) in order to remove platelets. Thereafter, PBMC were resuspended in culture medium RPMI 1640 (GIBCO BRL, Karlsruhe, Germany) containing 24 mM $NaHCO_3$ (Merck, Darmstadt, Germany), 10 mM HEPES (Roth, Karlsruhe, Germany), 1.6 mM glutamine (GIBCO BRL), 100 IU penicillin-streptomycin per ml (GIBCO BRL) and 10% fetal bovine serum (PAA Laboratories GmbH, 4020 Linz, Austria). Cells were used for the ELISPOT assay and CLA detection or frozen with culture medium containing 40% fetal bovine serum and 15% dimethylsulfoxide. PBMC were thawed at 37°C, and washed in culture medium if needed for further examinations.

Peptide synthesis and T2 cell loading The following HLA-A2 restricted peptide epitopes from melanocyte proteins were used for stimulation in the ELISPOT assay: LLDGTATLRL (Kawakami *et al*, 1995) VLYRYGSFSV (Kawakami *et al*, 1995), ITDQVPFSV (Kawakami *et al*, 1995), and YLEPGPVTA (Cox *et al*, 1994) from gp100. From tyrosinase we investigated MLLAVLYCL (Wolfel *et al*, 1994) and YMNGTMSQV (Wolfel *et al*, 1994), which can post-translationally be modified in YMDGTMSQV (Skipper *et al*, 1996). AAGIGILTV (Coulie *et al*, 1994), ILTVILGVL (Castelli *et al*, 1995), EAAGIGILTV (Kawakami *et al*, 1994; Romero *et al*, 1997) and its modified form ELAGIGILTV (Valmori *et al*, 1998) from MelanA/MART1. A mixture of viral epitopes was used as a positive control: CLGGLLTMV (Lee *et al*, 1993), LLDFVRFMGV (White *et al*, 1996), and GLCTLVAML (Steven *et al*, 1997) from Epstein–Barr virus (EBV), GILGFVFTL (Bednarek *et al*, 1991) from influenza and NLVPMVATV (Wills *et al*, 1996) from cytomegalovirus (CMV). Reaction against the peptide YLLPAIVHI (Hunt *et al*, 1992) from p72 helicase was assessed as unspecific stimulation. Peptides and their origin are listed in **Table I**.

Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the Fmoc/tBu strategy. Synthesis products were analyzed by high-performance liquid chromatography (System gold, Beckman Instruments, Munich, Germany) and MALDI-TOF mass spectrometry (G2025A, Hewlett-Packard, Waldbronn, Germany). Peptides of less than 80% purity were purified by preparative high-performance liquid chromatography. Peptides were dissolved in dimethylsulfoxide in a concentration of 10 mg per ml and further diluted to a final concentration of 1 mg per ml peptide with H_2O twice distilled.

For generation of stimulator cells, TAP-deficient HLA-A2-positive T2 (Salcedo *et al*, 1994) were incubated with 50 μg peptide per ml for 8 h in fetal bovine serum free culture medium. At the end of peptide incubation T2 cells were irradiated with 170 Gy (Gammacell 1000 Elite, MDS Nordion, Kanata, Ontario, Canada) and unbound peptide was removed by washing with RPMI 1640 containing 10% fetal bovine serum.

Table I. Peptides used for the detection of specifically reactive cytotoxic T cells

Peptide sequence	Protein	Position	Origin
KTWGQYWQV	gp100	154–162	Human
ITDQVPFSV	gp100	209–217	Human
YLEPGPVTA	gp100	280–288	Human
LLDGTATLRL	gp100	457–466	Human
VLYRYGSFSV	gp100	476–485	Human
MLLAVLYCL	Tyrosinase	1–9	Human
YMNGTMSQV	Tyrosinase	369–377	Human
AAGIGILTV	MelanA	27–35	Human
EAAGIGILTV	MelanA	26–35	Human
ELAGIGILTV	MelanA (modified)	26–35	Human
ILTVILGVL	MelanA	32–40	Human
YLLPAIVHI	p72 RNA helicase	146–154	Human
GLCTLVAML	BMLF-1	259–267	EBV
LLDFVRFMGV	EBNA-6	284–293	EBV
CLGGLLTMV	lmp-2	426–434	EBV
GILGFVFTL	Matrix protein	58–66	Influenza A
NLVPMVATV	pp65	495–503	CMV

ELISPOT assay Nitrocellulose 96 well plates (MAHA 45, Millipore, Bedford, MA) were coated with 50 μl per well human IFN-γ-specific antibody (20 μg per ml; Biosource, Camarillo, CA) diluted in coating buffer (35 mM sodium bicarbonate, 15 mM sodium carbonate and 3 mM sodium acid) for 3 h at 37°C. Unbound antibody was removed by four washing steps with PBS. Remaining protein binding sites of the nitrocellulose plate were blocked with culture medium for 1 h at 37°C.

PBMC (50,000 per well) and peptide-loaded T2 cells (70,000 per well) were co-cultured in triplicates in coated nitrocellulose 96 well plates. After 40 h of incubation at 37°C, 7% CO_2 cells were removed by washing seven times with PBS containing 0.05% Tween 20 (PBS/T). 50 μl of biotinylated human IFN-γ antibody (Biosource), diluted to 2 μg per ml in PBS containing 0.5% bovine serum albumin and 0.02% sodium azide, was used for the detection of bound IFN-γ. After 3 h unbound antibodies were removed by six washes with PBS/T and 50 μl of avidin peroxidase complex (ABC Vectastain-Elite Kit, Vector Laboratories, Burlingame, CA) was added. Two hours after addition of avidin peroxidase the plate was washed three times with PBS/T and three times with PBS. In the last washing step the complete plate was submerged in PBS. Subsequently, the reaction was developed with 3-amino 9-ethyl-carbazole (Sigma, St Louis, MO). After 5 min the color reaction was stopped by rinsing with water.

Computer-assisted video image analysis was used to count spots (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Pictures of wells were digitized by image software. The difference of contrast was checked for several parameters such as area, saturation, shape, slope, contrast, and color. Only spots corresponding to the parameters determined were counted.

The number of spots for the negative control peptide (YLLPAIVHI) was subtracted from the number of spots for peptides. The result is taken as the number of specifically reactive spots. If more spots were found in the control sample than in the test peptide samples negative values resulted. Values of the control collective consisting of 17 healthy persons were taken as reference values. The cut-off level was defined as the mean value of the control collective plus 2 SD. This means that 97.7% of the control persons were defined as the normal range of values and that 2.3% of control persons were expected to show elevated values. Cut-off level was calculated separately for each peptide tested. Higher values than defined by the cut-off level were regarded as representing specific PBMC reactivity.

Intracellular IFN-γ staining and fluorescence-activated cell sorter analysis The Cytofix/Cytoperm kit (PharMingen, San Diego, CA) was used for intracellular IFN-γ staining. Thawed cells were incubated for 3 h in a 24 well culture plate at 37°C. This leads to adherence of monocytes. The monocyte-free supernatant was used for further studies. Clusters of dead cells were filtered through a cell strainer (40 μm; Nylon, Falcon, Becton Dickinson, New York, NY). Peripheral blood

lymphocytes (10^6 per ml) were incubated in 4.5 ml polystyrene tubes (Greiner GmbH, Frickenhausen, Germany) with 10^6 peptide loaded T2 cells (2 h, 37°C). To stop IFN-γ secretion Golgi Stop (Cytofix/Cytoperm Kit, PharMingen) was added (0.7 μl per ml). After another 10 h, cells were washed with PBS and stained in fluorescence-activated cell sorter-buffer with 10 μg/100 μl CD8 tricolor antibody (CALTAG Laboratories, Burlingame, CA) and 20 μl fluorescein isothiocyanate labeled CLA antibody (rat IgM, PharMingen) for 30 min at 4°C. After removing unbound antibodies cells were fixed and permeabilized with 300 μl Cytofix/Cytoperm solution (Cytofix/Cytoperm Kit, PharMingen) for 20 min at 4°C. Cells were washed twice with 1 ml of Perm/Wash solution (Cytofix/Cytoperm Kit, PharMingen) and stained for 30 min in 100 μl Perm/wash solution with phycoerythrin-labeled mouse IFN-γ antibody (PharMingen) diluted to 1.5 μg per ml. Antibody was removed by two washes and cells were resuspended in PBS. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) with logarithmic amplification through a viable cell gate determined by forward and side scatter.

Culturing of PBL Fresh PBL (3×10^6) were incubated for 2 h in serum-free culture medium with 50 μg peptide per ml followed by co-culture with 10^6 autologous PBMC in a 24 well plate (Greiner GmbH, Frickenhausen, Germany). On day 4 culture medium was supplemented with 5% Lymphocult (Biotest, Germany) containing interleukin-2 and other unspecified T cell growth factors. T cell cultures were restimulated weekly with 3×10^6 peptide loaded autologous PBMC per well (31 Gy irradiated) and supplemented with Lymphocult. ELISPOT analysis was performed 7 d after restimulation.

Statistical testing The Wilcoxon Sum-of-Ranks (Mann–Whitney) test for comparing two unmatched samples was used for testing differences in the ELISPOT values between different subgroups of study subjects. $p < 0.05$ was statistically significant.

RESULTS

Detection of melanocyte-specific CD8+ T lymphocytes by ELISPOT in vitiligo patients Of the 32 patients tested 10 had actively progressive disease and in 22 the disease progression was moderate. In the PBMC of 11 patients (34%) T cells against the tested epitopes were detected. Seven of them had active disease. Four patients who reacted against the peptides had moderate disease progression (**Table II**).

The most frequently encountered immunodominant epitopes were the MelanA/MART1 peptide EAAGIGILTV and its modified form ELAGIGILTV. PBMC of nine patients reacted against one or both of these peptides, reaching 70–150 spots per 10^5 cells. In none of the healthy individuals spot frequencies of this magnitude were observed (**Fig 1**). Patients reacted significantly stronger against the epitope EAAGILGITV than healthy controls (Wilcoxon, $p = 0.0003$). There was also a significant difference between patients with active disease and patients with moderate disease progression (Wilcoxon, $p = 0.02$).

In two patients the peptide ILTVILGVL from MelanA/MART1 led to stimulation of T cells resulting in about 160 spots per 10^5 PBMC (**Fig 1**). This corresponded to 1–2% of the $CD8^+$ cells in these patients (**Fig 3**). Another patient also reacted against this epitope, albeit at a lower frequency.

Three patients reacted against peptides derived from the protein gp100. All of them also showed strong reactions against the MelanA/MART1 peptides EAAGIGILTV and its modified form ELAGIGILTV. Four patients were found reactive against tyrosinase peptides, they also reacted against the MelanA/MART1 peptides EAAGIGILTV or ILTVILGVL.

The patients showing high numbers of positive spots against one peptide, usually had specific reactivity against several other peptides. In patient 32, reactivity against all four peptides deriving from MelanA/MART1 and against three gp100 peptides and one tyrosinase peptide was found. Patient 8 showed reactivity against five peptides and patient 9 against four peptides. Altogether, six of 11 patients reacted against several peptides.

No reactivity in vitiligo patients was found against the gp100 peptide ITDQVPFSV. Weak reactivity in only one patient was observed against the gp100 peptide YLEPGPVTA and the tyrosinase peptide MLLAVLYCL. Only one of the control persons studied was found to show weak reactivity against the peptides YMNGTMSQV (tyrosinase) and ITDQVPFSV (gp100).

Although the reactivity against the peptide VLYRYGSFSV was weak, we found a significant difference between patients and healthy donors (Wilcoxon, $p = 0.004$). Interestingly, 11 of the 32 patients showed a strong reaction against the viral epitope mixture (EBV, influenza A, CMV) compared with the normal individuals (**Fig 1**, Wilcoxon, $p = 0.003$). In further experiments we stimulated the strongest reacting three control persons and three patients with CMV peptide in the same ELISPOT assay. In the patients a much higher frequency of CMV-specific T cells as compared with the control persons has been observed, suggesting that the higher reactivity against viral antigens was mainly directed against the CMV epitope (data not shown).

Course of the disease and development of specific T cell reactivity Patients who showed strong reactions in the ELISPOT assay and were classified as having active disease were followed in their clinical course and in their ELISPOT reactivity. In five patients acute attacks of vitiligo with disease progression were followed by phases of stable disease without recognizable progression, and a second blood sample has been examined by ELISPOT assay in these patients after 3–20 mo. Patient 32 had started ultraviolet B 311 nm irradiation therapy and had partial repigmentation of white spots. In ELISPOT assay a clear decrease of specific T cell reactivity could be demonstrated in each of the five patients (**Fig 2**).

Intracellular IFN-γ staining of PBMC confirmed positive reactivity Peripheral blood from the initial and second blood puncture for additional investigations was available from three patients reacting against melanocyte-specific peptides in the ELISPOT-assay and one patient not reacting in the ELISPOT. PBMC from these patients were tested for intracellular IFN-γ expression upon peptide stimulation. Patients 19 and 32 reacting strongly against ILTVILGVL (MelanA/MART1) in the ELISPOT were found to have a frequency of 1–2% of $CD8^+$ T cells against this peptide as shown by IFN-γ production. Patient 29 with a moderate ELISPOT frequency against the same peptide had less IFN-γ-positive $CD8^+$ T cells in intracellular IFN-γ staining. One of the ELISPOT-negative donors had no IFN-γ-positive $CD8^+$ T cells. **Figure 3** shows intracellular IFN-γ staining in patient 19 with blood samples taken in May and August.

Lack of CLA expression in PBL reacting against melanocyte epitopes For the same four patients whose T cells were measured by intracellular IFN-γ staining, expression of the CLA was examined. Peripheral blood $CD8^+$ T cells stimulated by melanocyte peptides to produce intracellular IFN-γ did not show any difference in CLA expression as compared with control peptides (data not shown).

DISCUSSION

Among the patients with actively progressive disease we detected reactive T cells in seven of 10 patients (70%). In patients classified as having moderately progressive disease only four of 22 patients (18%) had melanocyte peptide-reactive T cells. Patients without disease progression were not included in this study. We found T cells reactive to melanocyte peptides in about 34% of all HLA-A2-positive patients classified to have moderately to actively progressive vitiligo. Thus, the frequency of cytokine-producing T cells with specificity for melanocyte antigens is positively correlated with progressive vitiligo.

In a previous study (Ogg *et al*, 1998) addressing the T cell response against melanocyte epitopes tetramer staining was used. ELAGIGILTV (MelanA/MART1) -specific T cells were detected in seven of nine patients studied (Ogg *et al*, 1998) indicating this peptide to be immunodominant. Our study confirms ELAGIGILTV to be an immunodominant epitope also by ELISPOT, with most specific T cells also recognizing the artificial variant

Table II. Patient's characteristics, disease activity, and specific CTL reactivity[a]

Patient no.	Sex/ age	Duration of disease (mo)	Disease activity	% involved skin	ELAGIG ILTV (MART1)	EAAGI GILTV (MART1)	ILTVIL GVL (MART1)	AAGIGI LTV (MART1)	LLDGT ATLRL (gp100)	VLYRY GSFSV (gp100)	KTWGQ YWQV (gp100)	YLEPG PVTA (gp100)	ITDQV PFSV (gp100)	YMNGT MSQV (tyr.)	MLLAV LYCL (tyr.)
4	F/30	46	Active	40	+++	+++	–	–	–	–	–	–	–	–	–
5	F/29	54	Active	10	–	–	–	–	–	–	–	–	–	–	–
9	F/36	120	Active	20	+++	+++	–	–	+++	+	–	–	–	–	–
15	F/56	246	Active	10	–	+	–	–	–	–	–	–	–	–	–
18	F/47	54	Active	20	–	–	–	–	–	–	–	–	–	–	–
19	F/46	174	Active	3	–	–	+++	–	–	–	–	–	–	+	–
22	F/52	114	Active	20	–	+	–	–	–	–	–	–	–	–	–
27	F/21	48	Active	10	–	–	–	–	–	–	–	–	–	–	–
29	F/40	78	Active	80	–	–	++	+	–	–	–	–	–	+	–
32	M/45	54	Active	15	+++	+++	+++	++	+	++	+++	–	–	+++	–
1	F/35	174	Moderate	40	–	–	–	–	–	–	–	–	–	–	–
2	M/38	54	Moderate	15	–	–	–	–	–	–	–	–	–	–	–
3	M/37	210	Moderate	10	–	–	–	–	–	–	–	–	–	–	–
6	M/39	120	Moderate	15	–	–	–	–	–	–	–	–	–	–	–
7	F/55	120	Moderate	20	–	–	–	–	–	–	–	–	–	–	–
8	F/35	354	Moderate	15	++	+++	–	–	–	–	–	+	–	++	+
10	F/48	294	Moderate	15	–	–	–	–	–	–	–	–	–	–	–
11	F/21	174	Moderate	10	–	–	–	–	–	–	–	–	–	–	–
12	F/65	272	Moderate	20	–	–	–	–	–	–	–	–	–	–	–
13	F/41	282	Moderate	10	–	–	–	–	–	–	–	–	–	–	–
14	M/52	294	Moderate	15	–	+	–	–	–	–	–	–	–	–	–
16	F/64	294	Moderate	90	–	+	–	–	–	–	–	–	–	–	–
17	F/33	306	Moderate	20	–	+	–	–	–	–	–	–	–	–	–
20	F/34	186	Moderate	25	–	–	–	–	–	–	–	–	–	–	–
21	M/69	156	Moderate	10	–	–	–	–	–	–	–	–	–	–	–
23	F/57	54	Moderate	90	–	–	–	–	–	–	–	–	–	–	–
24	F/14	42	Moderate	15	–	–	–	–	–	–	–	–	–	–	–
25	M/43	54	Moderate	3	–	–	–	–	–	–	–	–	–	–	–
26	M/36	210	Moderate	15	–	–	–	–	–	–	–	–	–	–	–
28	F/51	174	Moderate	5	–	–	–	–	–	–	–	–	–	–	–
30	F/53	402	Moderate	75	–	–	–	–	–	–	–	–	–	–	–
31	F/49	90	Moderate	8	–	–	–	–	–	–	–	–	–	–	–

[a]Reactions with more spots than the 97.7 percentile (mean ± 2 SD) of controls was assessed as low response (+), reactions higher than mean ± 3 SD was assessed as moderate (++), and reactions with more spots than mean ± 4 SD as strong (+++) response.

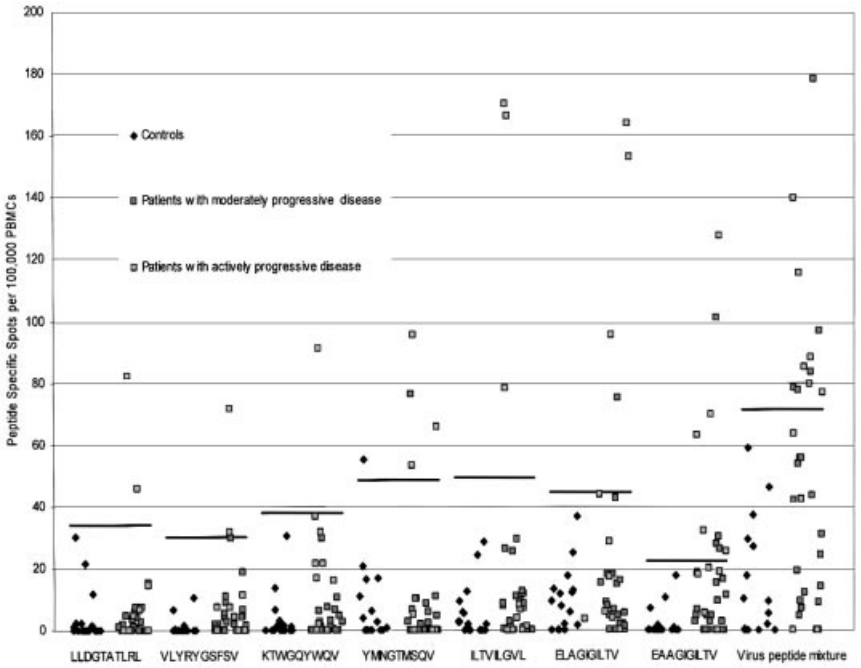

Figure 1. ELISPOT assay. The number of peptide-specific reactive spots are given. The background as seen for the control peptide YLLPAIVHI was subtracted in order to obtain the number of peptide-specific spots per 100,000 PBMC. Negative values are set to zero in this figure. Negative values were, however, used for the calculation of mean values for the control patients. The cut-off level was determined to be the mean ± 2 SD (97.7%) of the number of spots of control subjects. Background: Ges1=17, Ges2=6, Ges3=3, Ges4=1, Ges5=212, Ges6=48, Ges7=14, Ges8=39, Ges9=87, Ges10=255, Ges11=15, Ges12=176, Ges13=24, Ges14=36, Ges15=99, Ges16=126, Ges17=16, Pat1=9, Pat2=25, Pat3=106, Pat4=6, Pat5=Pat6=17, Pat7=2, Pat8=11, Pat9=55, Pat10=50, Pat11=10, Pat12=2, Pat13=5, Pat14=51, Pat15=15, Pat16=48, Pat17=4, Pat18=65, Pat19=57, Pat20=14, Pat21=56, Pat22=68, Pat23=11, Pat24=29, Pat25=13, Pat26=14, Pat27=23, Pat28=1, Pat29=72, Pat30=178, Pat31=13, Pat32=43, Pat33=1.

ELAGIGILTV. Only a smaller fraction of vitiligo patients (34% of total), however, was found to be reactive. In addition to ELAGIGILTV, we find ILTVILGVL from MelanA/MART1 as an additional antigen for HLA-A2-restricted CD8+ T cells in three of 32 vitiligo patients.

For the epitope EAAGIGILTV we found a significant difference between patients and healthy controls. It is an interesting observation that the frequency of melanocyte-specific T cells is likewise associated with disease activity. Classification of disease severity was done in our study in advance of the immunologic investigations and different investigators performed clinical assessment (C.C.C.) and ELISPOT assay examinations (K.S.L.). The judgement of disease stage could, therefore, not have been biased by knowledge of the ELISPOT results. We did not expect to detect these clear differences in melanocyte-specific T cell reactivity between patients classified as actively progressive (70%) and moderately progressive. Also the fact that specific T cell reactivity decreased in patients with stable vitiligo could mean that T cell reactivity is associated with disease activity.

We could identify functional T cells directed against the peptides in about 34% of our vitiligo patients, whereas Ogg *et al* (1998) found tetramer binding T cells in seven of nine patients (78%). This discrepancy may be due to one or more of the following three reasons: First, not all cells binding to tetramers might be functionally active and, therefore, negative in the ELISPOT assay. As we found IFN-γ producing cells it is likely that we detected antigen-responsive memory cells. Recent reports showed already that there are tetramer-positive cells in healthy donors that do not express CD45RO and could not produce IFN-γ in ELISPOT assay (Pittet *et al*, 1999). Furthermore, in one melanoma patient a tetramer-positive anergic T cell population has been described (Lee *et al*, 1999). These findings suggest that nonreactive melanocyte-specific T cells may be present in vitiligo patients. Second, the selection of study subjects in respect to the grade of disease activity may differ between both studies with a higher percentage of highly active vitiligo in the Ogg *et al* (1998) study. Third, the ELISPOT assay is not as sensitive as tetramer staining resulting in a lower percentage of positive T cells (Pittet *et al*, 1999).

Whereas Ogg *et al* (1998) found one of six healthy donors to be tetramer positive, only one of the 17 healthy controls in our study reacted weakly against two of the peptides as examined by the ELISPOT assay. This may likewise be explained by the higher sensitivity of the tetramer technique and the fact that tetramer binding does not demonstrate functional activity of the T cells and IFN-γ synthesis. After stimulation of T cells of healthy blood donors with professional antigen-presenting cells such as dendritic cells it is possible to generate MelanA-specific T cells (Jenne *et al*, 2000). Such results suggest that T cells against MelanA antigens rest in the blood of many healthy donors. The fact that we could detect such T cells in only one donor suggests that the frequency of these T cells is very low or that they are naive T cells. In order to improve the sensitivity of ELISPOT assay, we tried repeated *in vitro* stimulation of PBMC in cell culture with different peptides. This led to high ELISPOT numbers against ELAGIGILTV and EAAGIGILTV, but not for the other peptides (data not shown); however, after *in vitro* stimulation specific T cells against ELAGIGILTV and EAAGIGILTV were, likewise, found in two of five healthy donors. Therefore, we did not apply repeated *in vitro* stimulation with peptides further on in our experiments.

In five patients with active disease the subsequent course of disease was monitored and additional ELISPOT examinations have been performed. In all patients active phases of vitiligo were followed by phases of stable disease without recognizable progression. In all five patients we found 3–20 mo after first examination a clear decrease of specific T cell reactivity in the ELISPOT assay. One of these patients received ultraviolet B irradiation as a vitiligo treatment after his first blood puncture. In this patient, likewise, a clear decrease of ELISPOT reactivity could be demonstrated; however, this decrease was similar to the decrease in the other four patients not receiving ultraviolet B irradiation and, additionally, there was no clear decrease in his reactivity to viral epitopes. Therefore, we have no clear evidence for the involvement of ultraviolet B irradiation in decreasing melanocyte-specific T cell reactivity.

The CLA expression of peripheral blood CD8+ T cells stimulated by melanocyte peptides was, likewise, examined. CLA-positive CD8+ T cells were neither found in tested blood of the three vitiligo patients having actively progressive disease nor in the blood of one control individual. In the other patients no more blood from the first blood puncture was available for these investigations after performing ELISPOT examinations. Ogg *et al* (1998) reported that they have found a significant difference in CLA expression by the peptide-stimulated melanocyte-specific

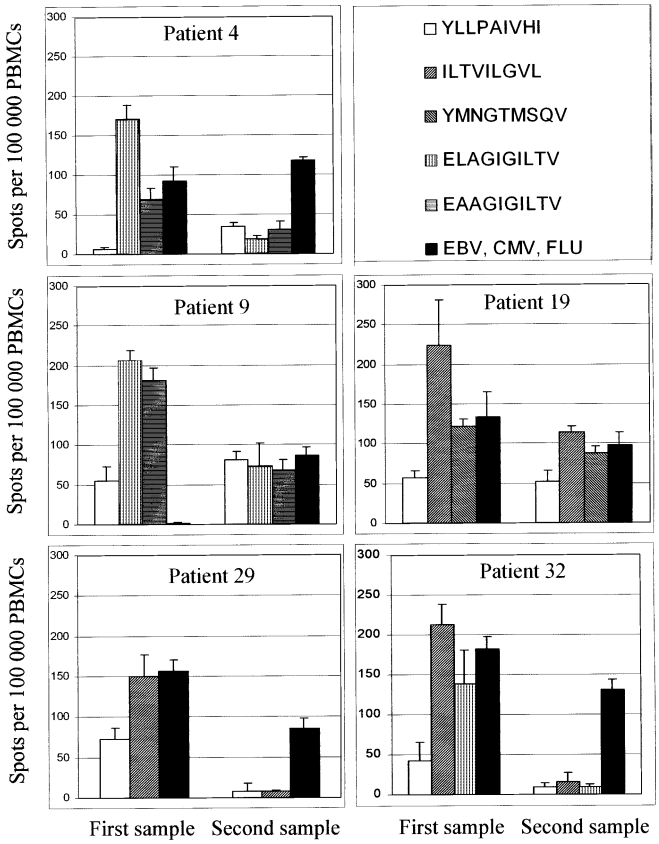

Figure 2. ELISPOT assay from patients with active disease measured at two different time points. Second blood sample was taken 3–20 mo later.

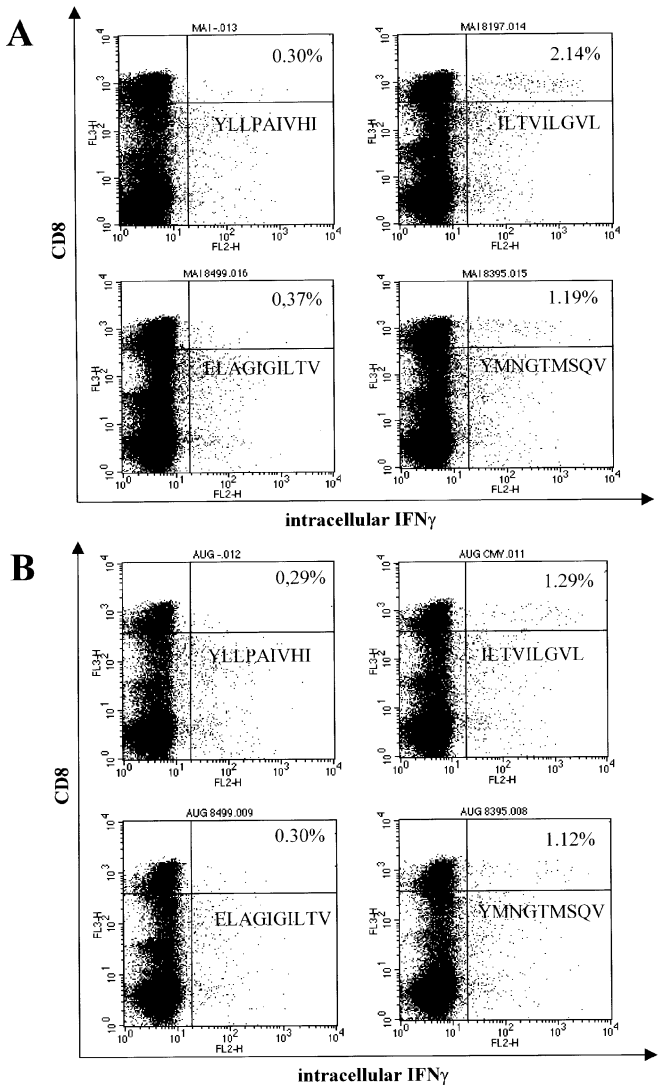

Figure 3. Representative dot plots of PBMC from patient 19 stained for CD8 and intracellular IFN-γ after peptide stimulation with MelanA/MART1 peptides ELAGIGILTV and ILTVILGVL, and tyrosinase peptide YMNGTMSQV. Cells were gated for forward and side scatter. Percentage is given for peptide-specific cells calculated from all $CD8^+$ cells. (*A*) Represents a blood sample taken in May 1999 and (*B*) a blood sample from August 1999.

CTL between patients and control individuals. Ogg *et al* (1998) believed that lack of the skin homing receptor on the surface of CTL might be a mechanism to prevent autoreactivity *in vivo* and to control peripheral tolerance. Our finding of lack of CLA expression of melanocyte-specific $CD8^+$ T cells does not support any correlation between the frequency or extent of the vitiligo and the CLA expression on the surface of $CD8^+$ T cells.

Several explanations may be considered for the low frequency of sensitized T cells in 66% of our vitiligo patients. First, epitopes other than the peptides used in this study may be attacked by T cells inducing melanocyte eradication. These epitopes could be different HLA-A2 restricted epitopes from the same molecules (MelanA/MART1, tyrosinase and gp100) or, likewise, from other molecules expressed by melanocytes, or these epitopes could be restricted to another major histocompatibility complex molecule, for example to HLA-B21 or HLA-Cw6, which were shown to be associated with vitiligo (al-Fouzan *et al*, 1995). Second, in a number of patients the disease may have not been active at the time point of study and T cells responding to the MelanA/MART1 or other melanocyte antigens may be absent from PBL in periods of stable disease. Third, sensitive T cells might be entrapped locally at the site of disease and cleared from PBL. Fourth, subgroups of vitiligo may exist that are not induced by activated T cells, but by other mechanisms destroying melanocytes, such as antibodies or natural killer cells (Naughton *et al*, 1983; Norris *et al*, 1988; Hann and Kim, 1995; Baharav *et al*, 1996; Fishman *et al*, 1997).

Interestingly, the vitiligo patients responded significantly stronger than control individuals to a mixture of viral antigens, i.e., the peptides CLGGLLTMV, LLDFVRFMGV, GLCTLVAML from EBV, the peptide GILGFVFTL from influenza and the peptide NLVPMVATV from CMV. This is not explained by a higher number of $CD8^+$ T cells in patients, as neither we (data not shown) nor others (Mozzanica *et al*, 1990) found a striking difference of $CD8^+$ cell abundance between vitiligo patients and healthy blood donors in fluorescence-activated cell sorter analysis. Instead, the higher frequency of virus-specific T cells in vitiligo patients point to a role of viral infection in the pathogenesis of the disease. CMV has been shown to enter the skin in vitiligo patients (Grimes *et al*, 1996). One may speculate that the immune response against the virus could lead to the damage of melanocytes and thus trigger vitiligo. Another possible explanation could be a direct destruction of melanocytes by CMV.

In conclusion, MelanA/MART1 is the dominant target (epitope EAAGIGILTV and epitope ILTVILVGVL) and tyrosinase and gp100 are additional targets for T cell autoimmune response in vitiligo patients. Melanocyte-specific reactive T cells were mainly found in actively progressive disease, and much less in patients with moderate disease. As not more than 70% of the patients with active vitiligo studied responded to those epitopes, additional antigens or presentation by other major histocompatibility complex molecules may play a part. The present findings support the hypothesis that vitiligo is a $CD8^+$ T cell-mediated autoimmune disease.

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