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Cytolytic T cell reactivity against melanoma-associated differentiation antigens in peripheral blood of melanoma patients and healthy individuals

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Antigenic peptides derived from several differentiation antigens of the melanocyte lineage were recently identified in human melanomas as targets for HLA-A2.1-restricted cytotoxic T lymphocytes (CTLs). To examine their potential role in tumour-directed immune responses *in vivo*, we determined CTL reactivity against seven antigenic peptides derived from the Melan A/MART-1, tyrosinase and gp100/Pmel17 antigens in the peripheral blood of 10 HLA-A2+ healthy controls and 26 HLA-A2+ melanoma patients. The influenza matrix peptide (GILGFVFTL) presented by HLA-A2.1 was used as a control peptide. CTL reactivity was assessed in a mixed lymphocyte 'peptide' culture assay. Reactivity against Melan A/MART-1-derived peptide antigens was readily detectable in both melanoma patients and controls. Reactivity directed against tyrosinase-derived peptide antigens was also detected in both melanoma patients and healthy individuals, but less frequently. A measurable response against gp100/Pmel17-derived antigens was found in 1/10 controls and in 1/26 of the melanoma patients. Reactivity against the influenza matrix peptide was common in both melanoma patients and controls. Our findings show that precursor CTLs against melanocyte differentiation antigens can be detected in peripheral blood of melanoma patients and healthy individuals. The pattern of CTL reactivity directed against melanoma-associated antigens does not seem to be altered in melanoma patients. Despite antigen-specific CTL reactivity, tumour growth was not prevented in melanoma patients and autoimmune phenomena were not detected in healthy individuals. It remains to be determined whether precursor CTLs recognizing melanocyte differentiation antigens can be activated by immunization and lead to effective tumour rejection *in vivo*.

Key words: cytotoxic T lymphocyte responses, melanoma-associated antigens

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Introduction

It is well established that human melanoma can be recognized by cytolytic T lymphocytes (CTLs).[1] Melanoma-associated antigenic peptides 9–10 amino acids long have been identified as target antigens for CTLs *in vitro* and *in vivo*. The first of these to be characterized was the MAGE gene family, which codes for CTL-defined antigens presented by HLA-A1 in melanomas and some other malignant tumours, but not in any normal tissues apart from the testis.[2–4] Tyrosinase, Melan A/MART-1, gp100/Pmel17 and gp75, which code for shared melanoma-associated antigens were then identified.[5–9] These antigens are also expressed in normal melanocytes during melanocyte differentiation and therefore classified as differentiation antigens.[5–8,10,11] Furthermore, several peptides derived from these differentiation antigens have been identified as targets for T-cell-mediated lysis in an HLA-A2.1-restricted fashion in melanomas and melanocytes.[10,2–17]

The simultaneous expression of differentiation antigens in most melanomas may render them suitable targets for *in vivo* immune responses that could result in tumour rejection.[12,18–20] Since approximately 49% of Caucasians are type HLA-A2+, the identification of potential tumour rejection antigens recognized in the context of HLA-A2.1 could open immunotherapeutic strategies of broad applicability.

The current challenge is to devise effective strategies to induce or augment a tumour-directed immune response by vaccination, using antigenic peptides that are known to be expressed by tumours and recognized by the immune system. Melanocyte differentiation antigens

such as tyrosinase, gp75, gp100/Pmel17 and Melan A/MART-1 may become potential targets for immune interventions in melanoma. For the development of active vaccination strategies, the presence of precursor CTLs that recognize the target antigen is a basic requirement. In this study we determined the pattern of CTL reactivity against peptide epitopes derived from Melan A/MART-1, tyrosinase and gp100/Pmel17 that have been identified as targets for CD8+ CTLs in melanomas and normal melanocytes in 26 HLA-A2+ patients with advanced melanoma and 10 HLA-matched healthy individuals.

Materials and methods

Cell cultures

Cell lines designated as SK were a generous gift from L. J. Old (Ludwig Institute for Cancer Research, New York). The stable melanoma cell clone SK29-MEL-1 was obtained by limiting dilution as previously described.[21] Epstein–Barr virus (EBV)-transformed B lymphocytes were generated from the same patient (SK29-EBV-B) and from a patient with renal cancer (MZ1257-EBV-B).[22] Tumour cell lines designated NW or MZ were established in our laboratory at II Medizinische Klinik, Krankenhaus Nordwest, Frankfurt, Germany. All tumour cell lines were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 10 mM Hepes buffer, 84 mg/l L-arginine, 584 mg/l L-glutamine, 10 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS). EBV-transformed B lymphocytes, SK29-EBV, MZ1257-EBV and the mutant cell line CEM × 721.174.T2 (T2) were maintained in RPMI 1640 medium supplemented with 10 mM Hepes buffer, 242 mg/l L-arginine, 50 mg/l L-asparagine, 300 mg/l L-glutamine, 10 IU/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids and 10% FCS.

Melanoma-associated peptides

Nona- or decamer peptides derived from Melan A/MART-1, tyrosinase, gp100/Pmel17 and the influenza matrix peptide were evaluated for spontaneous specific CTL responses from melanoma patients and healthy controls. Studies on Melan A/MART-1 epitope variants with one amino acid extension at position 26 of the protein sequence have revealed marked differences of the *in vitro* immunogenicity of peptides (A. van Pel, personal communication). Therefore, the immunogenic nonamer and the decamer with glutamic acid (E) at position 1 were evaluated for spontaneous CTL response. The tyrosinase protein contains two antigenic fragments at positions 1–9 and 368–376, both presented by HLA-A2. The second epitope was demonstrated to be more immunogenic after replacing the asparagine at position 3 with aspartic acid.

Mass spectrometric analysis has identified the latter variant to be a naturally processed peptide, produced by post-translational desamination.[23] The gp100/Pmel17-derived peptides are a nona- and a decapeptide at positions 280–288 and 457–466, respectively. The first epitope has also been identified through the use of mass spectrometry.[12] Peptides used in this study were synthesized by Saxon Biochemicals (Hannover, Germany) and UCB Bioproducts (Braine-l'Alleud, Belgium). All peptides were solubilized in 20% DMSO and phosphate-buffered saline. The sequences of the peptides used in the experiments are listed in Table 1.

Mixed lymphocyte peptide culture (MLPC)

Frozen peripheral blood lymphocytes (PBLs) were thawed and washed in RPMI 1640 supplemented with 10% human serum, 50 mg/l L-asparagine, 242 mg/l L-arginine and 300 mg/l L-glutamine. PBLs were seeded in 48-well plates (1×10^6 cells/well) in 0.5 ml medium. Remaining PBLs, irradiated at 3,000 rad, were resuspended in 1 ml serum-free RPMI supplemented with β2-microglobulin (2.5 μg/ml) and peptide (10 μg/ml) and incubated for 1 h at room temperature. Stimulating cells were then washed once and added to the MLPC at 1×10^6 cells/well. Interleukin-2 (25 IU/ml; Biotest Pharma, Dreieich, Germany) was added on day 3. At day 7 responder cells were harvested, washed and transferred to 24-well plates. Peptide was added at 1 μg/ml. At day 14 responder cells were harvested for the cytotoxicity assay.

Analysis of CTL reactivity was performed after a relatively short culture period of 2 weeks. Theoretically, this may lead to an underestimation of peptide immunogenicity. However, our experience with large numbers of MLPC assays has shown that a CTL-dependent target lysis of >20% after 2 weeks is usually increased by more rounds of re-stimulation. In contrast, target lysis of

Table 1. Melanoma-associated peptides

Peptide	Antigen no.	Sequence
Melan A	1	AAGIGILTV
	2	EAAGIGILTV
Tyrosinase	3	MLLAVLYCL
	4	YMDGTMSQV
gp100/Pmel17	5	YLEPGPVTA
	6	LLDGTATLRL
Influenza matrix	7	GILGFVFTL

< 20% after 2 weeks was not amplified by more rounds of re-stimulation in > 90% of MLPC assays performed.

Cytotoxicity assays

For determination of cytotoxicity against peptide-pulsed targets, 10^6 T2 cells were incubated with 10 μl of monoclonal antibody MA 2.1[24] in ascites and labelled with 100 μCi of Na[^{51}Cr]O_4, washed once and resuspended in serum-free medium supplemented with 1 μg/ml of peptide. Cells were incubated for 1 h at room temperature. Responder cells were added to the peptide-pulsed target cells at effector : target ratios of 90, 30, 10, 3 and 1. Unlabelled K562 cells were added to responder cells at a ratio of 80 : 1 to block natural killer cell activity. T2 cells without peptide were used as targets to exclude CTL reactivity towards T2 cells alone. [^{51}Cr]-labelled targets (1000 cells/well) and 50 μl of responder CTL suspension were incubated in conical 96-well microtitre plates (Greiner, Nürtingen, Germany) for 4 h in a water-saturated atmosphere with 5% CO_2 at 37°C. The plates were centrifuged at 200 ***g*** for 5 min, 100 μl of supernatant was removed and radioactivity was measured in a γ-counter.

The percentage of specific ^{51}Cr release was determined by the formula:

$$\%\ \text{specific}\ ^{51}\text{Cr release} = \frac{(\text{experimental}\ ^{51}\text{Cr release} - \text{spontaneous}\ ^{51}\text{Cr release})}{\text{maximum}\ ^{51}\text{Cr release} - \text{spontaneous}\ ^{51}\text{Cr release})} \times 100$$

Maximum ^{51}Cr release was obtained by adding 100 μl Nonidet P40 1% (Sigma) to labelled target cells. Spontaneous ^{51}Cr release ranged from 5 to 10% of total counts incorporated into cells. ^{51}Cr release from unlabelled T2 cells ranged from 3 to 10%.

A CTL response was considered to be positive with a peptide-specific lytic activity exceeding 20% at an E:T ratio of 90 : 1. Results from preliminary test series indicated that a target lysis of 20% at this ratio was a suitable cut-off to eliminate non-specific background activity.

Melanoma patients and healthy individuals

Twenty-six HLA A2 patients with metastatic melanoma and 10 HLA-A2 + healthy controls were examined for their CTL reactivity against a panel of HLA-A2-restricted antigenic peptides derived from differentiation antigens. Any previous chemotherapeutic or cytokine-based intervention had been discontinued for more than 8 weeks in melanoma patients. All individuals included in this study gave formal consent for 100 ml blood to be drawn for the determination of CTL reactivity against the various peptide antigens.

Results

We report here the results on CTL responses against peptides derived from the differentiation antigens Melan A/MART-1, tyrosinase and gp100/Pmel17, determined in the peripheral blood of melanoma patients and healthy individuals. These self-peptides have been previously identified as targets for CTLs that had been isolated from the blood of single HLA-A2 + melanoma patients and healthy controls. In the present study, the pattern of baseline CTL reactivity against different antigenic targets was examined in 26 melanoma patients and 10 healthy individuals. Peptide-specific CTL responses evoked in the MLPC assay *in vitro* reflect the repertoire of inducible precursor CTLs. The identification of specific CTL precursors sets the basis for the development of individual tumour vaccines.

CTL reactivity against melanoma-associated peptides

In healthy controls. Ten HLA-A2 + healthy controls were evaluated for CTL activity against melanoma-associated peptides. Irradiated autologous PBLs pulsed with peptides served as stimulating antigen-presenting cells and peptide-sensitized T2 cells as targets for the measurement of CTL responses *in vitro*. The pattern of spontaneous CTL activity in controls is presented in Table 2a. As previously reported, measurable CTL activity (> 20% specific lysis of peptide targets) was detected against peptides derived from Melan A/MART-1 [(E)AAGIGILTV], tyrosinase (MLLAVLYCL, YMDGTMSQV) and gp100/Pmel17 (LLDGTATLRL) and against the influenza matrix peptide (GILGFVFTL). Melan A/MART-1 peptides were recognized in two of the 10 healthy controls. Tyrosinase-derived epitopes were recognized in one control, as was the second epitope of the gp100/Pmel17 antigen (LLDGTATLRL). The peptide-specific CTL responses obtained in the MLPC assay were also tested on Melan A/MART-1- and tyrosinase-expressing tumour cell lines (SK29-MEL-1, SK29-MEL, NW16-MEL). A significant cross-lysis of melanoma cell targets was demonstrated (Table 3). The influenza matrix peptide was recognized by 5/10 controls. Reactivity against melanoma-associated epitopes and the influenza matrix peptide showed no correlation (Table 2a).[25]

In melanoma patients. Twenty-six HLA-A2 + melanoma patients with advanced disease were evaluated for CTL activity against melanoma-associated peptides. The results are presented in Table 2b. Measurable CTL activity (> 20% specific lysis of peptide targets) was detected against the natural Melan A/MART-1-derived peptides

Table 2a. Cytolytic T lymphocyte reactivity against melanoma-associated peptides in healthy controls

	Reactivity (% cytotoxicity) for each peptide at each effector:target ratio																											
	Melan A/MART-1								Tyrosinase								gp100/Pmel17								Influenza matrix			
	AAGIGILTV				EAAGIGILTV				MLLAVLYCL				YMDGTMSQV				YLEPGPVTA				LLDGTATLRL				(GILGFVFTL)			
	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1
NW19	37	45	36	14	39	37	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	43	40	25	10
NW20	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0
NW21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	32	43	22
NW22	3	14	14	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	4	4	1
NW23	0	0	0	0	34	42	28	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	54	33	20
NW25	0	0	0	0	3	2	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	0
NW26	2	3	3	1	0	1	0	0	0	0	0	0	2	0	0	0	4	0	0	3	0	2	4	3	0	0	6	4
42380	0	7	4	0	14	5	4	2	0	0	0	0	0	0	0	0	0	0	0	0	22	8	13	4	96	91	74	43
42363	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	67	66	45	21
42374	0	0	0	0	—	—	—	—	46	40	18	6	0	0	0	0	0	0	0	0	0	0	0	0	89	79	52	33

Cytotoxicity was determined in standard 4 h ^{51}Cr-release assays on T2 cells pulsed with each peptide at the various effector:target ratios.

[(E)AAGIGILTV], the tyrosinase-derived epitopes (MLLAVLYCL and YMDGTMSQV) and one of the gp100/Pmel17-derived epitopes (YLEPGPVTA). Melan A/MART-1 peptides were recognized by CTLs of seven of the 26 patients. One of these seven patients showed CTL reactivity against the Melan A/MART-1-derived nonamer (AAGIGILTV) exclusively, while the remaining six showed CTL reactivity against both with comparable intensity. Tyrosinase peptides were recognized by three patients. CTL reactivity against the tested gp100/Pmel17-derived peptides was detected in one patient. The peptide-specific CTL responses obtained in the MLPC assay were also tested on Melan A/MART-1- and tyrosinase-expressing tumour cell lines (SK29-MEL-1, SK29-MEL, NW16-MEL). A significant lysis of melanoma cell targets was demonstrated (Table 3). CTL responses against the influenza peptide were detected in 18 patients. As for healthy controls, there was no correlation between reactivity detected against melanoma-associated peptide epitopes and against the influenza control peptide (Table 2b).

Discussion

The identification of shared target antigens for T-cell-mediated lysis in melanoma and other tumours set the basis for the development of antigen-directed vaccines. The presence in melanoma patients of multiple precursor CTLs that recognize these antigens would open perspectives for the construction of polyvalent active and adoptive immunotherapeutic strategies.

Previous investigations have demonstrated that anti-melanoma CTLs can be generated by *in vitro* stimulation with single antigenic peptide epitopes derived from differentiation antigens from PBLs of melanoma patients and healthy individuals.[26–28] The present report is the first more detailed pattern analysis of CTL reactivity against peptide epitopes derived from Melan A/MART-1, tyrosinase and gp100/Pmel17 detectable in PBLs of melanoma patients and healthy controls.

Our findings demonstrate that a measurable CTL reactivity against one or more melanoma-associated antigens is detectable *in vitro* in PBLs of melanoma patients and healthy controls. The naturally processed Melan A/MART-1-derived peptides were recognized more frequently than tyrosinase and gp100/Pmel17 epitopes. This may be due to an increased antigenicity of Melan A/MART-1-derived peptides in the MLPC assay. Alternatively, the pattern of CTL reactivation *in vitro* may reflect the presence of inducible precursor CTLs *in vivo*. Theoretically, the portion of specific CTLs present in the individual T cell repertoire that has escaped clonal deletion may be stimulated by appropriate antigen presentation *in vitro*. In this way, primary activation of CTLs that had not encountered 'cryptic' epitopes *in vivo* may result. On the other hand, CTLs that had already been stimulated *in vivo* may be reactivated by a secondary response *in vitro*.[29] In the MLPC assay, primary CTL responses cannot be discriminated from secondary reactivation *in vitro*. However, peptide-pulsed PBLs used as stimulators are considered to be less potent than 'professional antigen presenting cells' (i.e. dendritic cells). It may be that only previously *in vivo* activated CTLs providing an increased precursor frequency can be re-stimulated in this assay. In the limited number of individuals observed, the pattern of CTL reactivity in melanoma patients was not altered compared with the pattern in healthy controls. This suggests that the baseline CTL reactivity against differentiation antigens is usually not affected

Table 2b. Cytolytic T lymphocyte reactivity against melanoma-associated peptides in melanoma patients

	Reactivity (% cytotoxicity) for each peptide at each effector:target ratio																											
	Melan A/MART-1								Tyrosinase								gp100/Pmel17								Influenza matrix			
	AAGIGILTV				EAAGIGILTV				MLLAVLYCL				YMDGTMSQV				YLEPGPVTA				LLDGTATLRL				(GILGFVFTL)			
	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1
NW13	14	5	2	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	19	11	4	3
NW16	57	34	18	12	39	24	10	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	21	17	13	8
NW15	0	0	0	0	0	0	0	0	0	1	0	0	41	16	9	3	17	7	2	0	0	1	0	0	45	27	18	3
NW12	2	0	0	0	13	3	0	2	–	–	–	–	0	0	0	0	0	0	0	0	2	1	0	0	60	43	21	5
NW14	0	0	0	0	7	3	3	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	25	18	15	6
NW24	47	35	26	11	30	21	15	7	24	27	15	7	0	0	0	0	0	0	0	0	0	0	0	0	54	31	18	4
MZ11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MZ13	49	29	18	6	0	0	0	0	16	8	5	4	0	0	0	0	0	0	0	0	0	0	0	0	75	59	43	19
MZ20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	7	3	1
SK29	57	46	31	14	48	31	16	3	0	0	0	0	0	0	0	0	0	0	0	0	–	–	–	–	3	1	0	0
MZ21	0	0	0	0	2	1	0	0	2	2	1	0	0	0	0	0	13	8	5	1	0	0	0	0	84	59	36	14
MZ22	17	13	8	1	24	18	12	4	32	22	15	7	8	5	2	0	27	18	11	4	0	0	0	0	29	23	11	3
MZ23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30	17	10	2
MZ24	9	3	1	0	11	4	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	24	15	9	2
MZ25	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	38	19	12	2
MZ26	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	1	0	0	26	16	8	1
NW28	0	0	0	0	6	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34	17	4	0
NW29	12	5	1	1	24	16	7	2	7	3	1	1	14	6	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NW30	10	6	2	1	8	1	0	0	4	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
NW33	23	18	14	9	11	8	4	1	9	3	1	1	7	2	0	0	14	3	1	0	13	6	2	0	38	15	6	0
NW36	0	0	0	0	7	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	7	2	1	0
NW37	13	4	1	1	0	0	0	0	6	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	33	21	6	0
NW39	8	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	–	–	–	46	26	12	1
NW40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36	21	8	1
NW41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	33	25	9	3
NW42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	52	36	25	15

Cytotoxicity was determined in standard 4 h ^{51}Cr-release assays on T2 cells pulsed with each peptide at the various effector:target ratios.

by the presence of a growing tumour, at least in terms of the pool of circulating lymphocytes.

Melan A/MART-1, tyrosinase, gp100/Pmel17 and gp75 are self-antigens that are expressed during the differentiation of normal melanocytes. It was previously demonstrated that cultured melanocytes as well as HLA-matched melanoma cells are efficiently lysed by CTL clones derived from melanoma patients. This suggests that self-antigens may become targets for cellular immune responses mediating tumour rejection as well as autoimmune reactions.[29] Symptoms of autoimmunity, however, were not detected in those healthy controls

Table 3. Cross-reactivity of cytolytic T lymphocytes from one healthy control and three melanoma patients against T2 cells pulsed with MelanA/MART-1- and tysosinase-derived peptides and melanoma cell lines expressing these antigens

	Reactivity (% cytotoxicity) at each E:T ratio																			
	Peptide								Cell line											
	Melan A/MART-1 (AAGI-GILTV)				Tyrosinase(MLLAVLYCL)				SK29-MEL-1				SK29-MEL				NW16-MEL			
	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1	90	30	10	1
NW19[a]	37	45	36	14	–	–	–	–	29	21	13	7	31	23	11	5	16	9	4	0
NW16	57	34	18	12	–	–	–	–	42	37	22	12	40	33	19	8	39	23	14	10
NW24	–	–	–	–	24	27	15	7	38	32	24	12	26	17	12	5	31	19	11	3
MZ22	–	–	–	–	24	18	12	4	21	13	6	0	24	16	8	1	18	12	6	0

who displayed measurable CTL responses to differentiation antigens. This may be due to down-regulated expression of antigens and co-stimulatory signals in normal tissues. Vitiligo has occasionally been observed in melanoma patients showing tumour regression or subsequent to adoptive transfer of tumour infiltrating lymphocytes specific for tyrosinase-, gp100/Pmel17- and gp75-derived antigens.[9,17,30] This indicates that large amounts of activated CTLs may elicit cellular immune reactions in tissues of low immunogenicity.

The apparent lack of an effective response against a growing tumour in the presence of measurable tumour-antigen-specific CTL reactivity in melanoma patients may be the consequence of an altered expression of target antigens in tumour tissues. Low density of peptide–major histocompatability complex (MHC) complexes, antigen-loss variants, inadequate expression of co-stimulatory signals and impaired antigen presentation caused by other surface molecules or aberrant structures of antigen-presenting molecules are known mechanisms of escape from efficient T cell interactions and may account for a loss of immunogenicity in melanomas.[29,31] It remains to be investigated whether potent vaccines can activate silent CTLs and lead to T-cell-dependent tumour rejection *in vivo*.

A number of different antigenic determinants expressed on the surface of growing tumour cells have been identified as targets for CTLs of different specificity. Even within one antigenic protein several epitopes at different positions may constitute peptide–MHC complexes of distinct immunogenicity.[11,14–16,18] Simultaneous immunization against several antigens may activate different precursor CTLs of the individual T cell repertoire. Depending on the pattern and density of antigen expression, a polyvalent activation of specific CTLs may not only lead to efficient destruction of tumour cells, but should also prevent potential escape of single-antigen-loss variants. Antigen expression in tumour tissues may be altered at different stages of the disease and may show distinct patterns in primary tumours and metastases.[32] With the assessment of antigen expression by mRNA–polymerase chain reaction and monoclonal-antibody immunohistochemistry, individual targets for potential immune intervention may be identified.[33,34] The determination of spontaneous CTL reactivity against these antigens may define the precursor pool of CTLs that have escaped deletion and that may be activated *in vitro* for adoptive immunotherapy or *in vivo* by potent vaccines.

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