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## **Induction and analysis of antigen-specific T cell responses in melanoma patients and animal model**

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## **CHAPTER 1**

# On the Role of Melanoma-Specific CD8<sup>+</sup> T-Cell Immunity in Disease Progression of Advanced-Stage Melanoma Patients.

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### **Abstract**

Cytotoxic T-cell immunity directed against melanosomal differentiation antigens is arguably the best-studied and most prevalent form of tumor-specific T-cell immunity in humans. Despite this, the role of T-cell responses directed against melanosomal antigens in disease progression has not been elucidated. To address this issue, we have related the presence of circulating melanoma-specific T cells with disease progression and survival in a large cohort of patients with advanced-stage melanoma who had not received prior treatment. In 42 (68%) of 62 patients, melanoma-specific T cells were detected, sometimes in surprisingly large numbers. Disease progression during treatment was more frequent in patients with circulating melanoma-specific T cells, and mean survival of patients with circulating melanoma-specific T cells was equal to the survival of patients without melanoma-specific T cells. These data suggest that the induction of melanosomal differentiation antigen-specific T-cell reactivity in advanced stage melanoma is a late event most likely due to antigen load and spreading and is not accompanied by a clinically significant antitumor effect. These melanoma-specific T cells may be functionally distinct from T cells raised during spontaneous regression or upon vaccination.

## Introduction

Melanoma is considered one of the most immunogenic tumors. Spontaneous remissions occasionally occur in melanoma patients albeit infrequently and immunotherapy-induced remissions correlate with autoimmune skin depigmentation<sup>(1, 2, 3, 4)</sup>. These phenomena have, at least in part, been attributed to a cellular immune response, because the presence of tumor-infiltrating T lymphocytes in primary melanoma and in melanoma lymph node metastases are independent positive prognostic factors<sup>(5, 6)</sup>.

In past years, extensive efforts to identify target molecules for melanoma-reactive T cells have resulted in the identification of a large set of melanoma-associated antigens<sup>(7)</sup>. These antigens can be classified as melanocyte lineage-specific antigens (MART-1/Melan-A, tyrosinase, gp100) and antigens derived from genes expressed in testis and a variety of cancers (including MAGE-family, NY-ESO-1, PRAME). Melanocyte lineage antigens are expressed in a large fraction of melanomas, and a substantial number of epitopes from these antigens that are recognized by cytotoxic T cells have been mapped<sup>(8)</sup>. With the aid of soluble tetramerized MHC complexes containing these epitopes, melanosomal antigen-specific CD8<sup>+</sup> T cells have been detected in peripheral blood from melanoma patients<sup>(9)</sup>. Large expansions of these T cells are primarily observed in the peripheral blood or tumor-infiltrated lymph nodes from stage III and particularly stage IV melanoma patients. In patients with tumors that are confined to the primary site, such as in stage I and II melanoma, MHC tetramer-positive T cells have been found, but at significantly lower frequencies<sup>(10)</sup>. Despite the presence of naturally occurring melanoma-specific T-cell immunity in a large proportion of melanoma patients, spontaneous remissions in this group of patients are extremely rare and it is currently unclear what the significance is of spontaneous melanoma-specific T-cell immunity for these patients.

## Patients & Methods

### *Patients.*

Peripheral blood samples were taken from 62 HLA-A\*0201 advanced-stage melanoma patients (stage IV) at the time that they had been diagnosed with metastatic melanoma. None of the patients had undergone prior treatment for metastatic melanoma. Patients were treated with standard chemotherapy with or without cytokine therapy mostly as part of a clinical trial<sup>(11)</sup>, as indicated in Table 1

### *MHC Tetramers.*

MHC class I tetrameric complexes were prepared as previously described with minor modifications<sup>(12)</sup>. HLA-A2.1-peptide complexes were generated with the following five antigenic peptides: (a) influenza-A matrix<sub>58-66</sub>; (b) GILGFVFTL, MART-1<sub>26-35</sub>; (c) ELAGIGILTV (A27L variant<sup>13</sup>); (d) tyrosinase<sub>368-376</sub>, YMDGTMSQV (N370D variant<sup>14</sup>); and (e) gp100<sub>280-288</sub>, YLEPGPVT (A288V variant<sup>15</sup>). MHC class I-peptide complexes were subsequently purified, biotinylated by biotin

ligase, purified and converted to tetramers by the addition of phycoerythrin-labeled streptavidin (Molecular Probes), and stored at  $-20^{\circ}\text{C}$  in 16% glycerol/0.5% BSA.

#### *MHC-Tetramer Staining.*

It has previously been shown that CD8 can play a critical role in the binding of MHC tetramers to antigen-specific T cells<sup>(16, 17, 18)</sup>. In particular, in case of low-affinity T-cell receptors, the simultaneous binding of CD8 to the MHC  $\alpha 3$  domain is necessary to obtain a sufficiently stable complex between MHC tetramers and antigen-specific T cells, and in such cases, costaining with CD8 can block MHC tetramer binding<sup>(16, 17, 18)</sup>. In line with this, we observed diminished MHC tetramer staining of a tyrosinase-specific T-cell clone in the presence of CD8 monoclonal antibody. Likewise, a large fraction of the tyrosinase-specific CD8<sup>+</sup> T cells in the peripheral blood of advanced-stage melanoma patients became undetectable by MHC tetramer staining in the presence of CD8 monoclonal antibody (data not shown). To overcome this issue, peripheral blood mononuclear cells (PBMCs) were stained with a set of lineage (lin) marker antibodies specific for B-cells (CD19), natural killer cells (CD16), monocytes (CD14) granulocytes (CD13), and CD4<sup>+</sup> T cells (CD4), and the lin<sup>negative</sup> lymphocyte subset was used for analysis. Lin<sup>negative</sup> lymphocytes are >95% CD8-positive and HLA-A2.1/tyrosinase tetramer-positive cells within the lin<sup>negative</sup> population are likewise >95% CD8-positive, thereby validating this strategy (Fig. 1)

Thawed PBMC samples were incubated overnight at  $37^{\circ}\text{C}$  in Iscove's medium with 10% FCS to recover PBMCs and eliminate apoptotic cells (9). After washing, the cells were incubated for 5 min in cold PBS with 0.5% BSA and 1% normal mouse serum to block Fc-receptors. Two million cells per sample were incubated for 10 min with 2  $\mu\text{g}/\text{ml}$  phycoerythrin-labeled MHC-tetramer at  $37^{\circ}\text{C}$ <sup>(19)</sup>. CD8<sup>+</sup> T cells were negatively selected by staining with a large set of FITC-labeled lineage marker antibodies (CD4, CD13, CD14, CD16, CD19; Becton-Dickinson). Cells were stained with propidium iodide to be able to gate out dead cells. Samples were analyzed by flow cytometry using a FACScalibur and Cell Quest software (Becton Dickinson). Forward and side scatter parameters were used to define lymphocyte populations.

Background and detection limits were separately established for each tetramer by staining of PBMCs from four HLA-A2-negative individuals with phycoerythrin-labeled MHC-tetramers and the panel of FITC-labeled antibodies. If more than 50,000 CD3<sup>+</sup>/CD8<sup>+</sup> cells could be analyzed by flow cytometry in the HLA-A2-positive samples, the mean percentage of MHC-tetramer positive cells +3x SD of four HLA-A2-negative PBMC samples was used as the detection limit for positive MHC-tetramer staining. If less than 50,000 CD3<sup>+</sup>/CD8<sup>+</sup> cells could be analyzed, the mean percentage +6x SD was used as the detection limit.

#### *Immunohistochemistry.*

Formalin-fixed paraffin-embedded material of melanoma metastases was available from 17 of 62 patients. Sections (4- $\mu$ m) were stained for MART-1 (Ab-3; Neomarkers; 1:250), tyrosinase (clone T311; Neomarkers; 1:100), gp100 (HMB-45; DAKO; 1:200) or HLA-A (HC-A2; generously donated by J. Neefjes, Netherlands Cancer Institute, Amsterdam; 1:400). For all stainings except for gp100, microwave antigen retrieval in citrate buffer was performed. Stainings were performed with standard alkaline-phosphatase three-step immunohistochemistry.

#### *Statistics.*

End point of the study was overall survival, measured from start of treatment until death or last follow-up. Patients alive at the time of analyses were censored. Survival estimates and curves were made with the Kaplan-Meier technique, and differences between groups were tested by a log-rank test. Cox proportional hazard analysis was used to estimate the size of the effect (hazard ratio) with 95% confidence intervals. The relation of MART-1 with response (in three categories) was tested by a Cochrane-Armitage trend test. Wilcoxon test was used to perform a statistical analysis on the phenotypical characteristics that distinguish naïve and effector/memory MART-1-specific T cells.

## **Results**

#### *Patients.*

Between 1998 and 2002, 62 HLA-A\*0201-positive patients were enrolled (Table 1). All patients had advanced-stage melanoma (stage IV) with a median organ involvement of 2 (range 1–5). The majority (42 of 62) of the patients had visceral metastases, mostly in lung and/or liver. Nonvisceral metastases (20 of 62) were most often located in lymph nodes and subcutis. Five patients were treated with standard dacarbazine (800 mg/m<sup>2</sup>, every 3 weeks), 10 patients were treated with temozolomide (200 mg/m<sup>2</sup> for 5 days, every 4 weeks) and 47 patients were treated with combined chemobiotherapy (temozolomide for 5 days, triple cytokine interleukin 2, interferon- $\alpha$ , granulocyte macrophage colony-stimulating factor for 12 days, every 4 weeks). The latter two treatments were given as part of Phase II and III clinical trials.

#### *Analysis of Patient-Derived Peripheral Blood Samples.*

Peripheral blood samples were obtained before initiation of therapy and were analyzed for the presence of melanoma-specific CD8<sup>+</sup> T cells (Fig. 2). In 33 patients (53%) MART-1-specific CD8<sup>+</sup> T cells were detectable, and 17 patients (27%) displayed expansions of tyrosinase-specific CD8<sup>+</sup> T cells. Eight of these latter patients also had MART-1-specific T cells. Only 1 patient tested positive for gp100-specific CD8<sup>+</sup> T cells; this patient also had high numbers of circulating MART-1-specific T cells (18% of total CD8<sup>+</sup> T-cell pool). In the vast majority of samples that contained tyrosinase-specific T cells, these CD8<sup>+</sup> T cells were clearly detectable when counterstained using the panel of lineage marker antibodies but were difficult to detect when costaining with anti-CD8 antibody was performed. In

line with this, the avidity of tyrosinase-specific T cells appears lower than that of MART-1- or gp100-specific T cells, as judged from the intensity of MHC tetramer staining. As a control, influenza A virus-specific CD8<sup>+</sup> T cells could be detected in 70% of patients analyzed (n = 36). In the majority of patients [27 (64%) of 42] with MHC tetramer<sup>+</sup> T cells, the frequencies of melanoma-specific T cells in peripheral blood were above 1:1000 CD8<sup>+</sup> T cells, ranging from 1:6 to 1:900.

MART-1-specific CD8<sup>+</sup> T cells have been detected in the peripheral blood of healthy individuals in frequencies up to 1 of 1,000<sup>(20)</sup>. Data from Dutoit et al.<sup>(21)</sup> indicate that these populations are a direct reflection of a high thymic output of T cells with this specificity, and, in line with this, these MART-1-specific T cells display a naïve phenotype. To establish whether the MART-1-specific T-cell populations detected in our cohort arose through antigen-driven expansion, the MHC tetramer-positive CD8<sup>+</sup> T cell subset was analyzed directly *ex vivo* for cell surface expression of naïve-, effector-, and memory-type-associated markers. In humans, the CD45RA and CD45RO surface antigens have been used to identify naïve and memory T cells, respectively, and costaining for CD27 expression may be used to distinguish between effector cells and naïve or memory T cells<sup>(22)</sup>. Using the Wilcoxon test, we found two statistically distinct patterns of cell surface expression (Table 2). In patients with low but detectable frequencies (< 0.2%) of MART-1-specific T cells, expression of CD45RA and CD27 were high (P = 0.033 and P = 0.0015, respectively); and expression of CD45RO was low (P = 0.0004). This pattern of cell surface expression reflects a naïve phenotype. In contrast, in patients with high frequencies (> 0.5%) of MART-1-specific T cells CD45RO expression was high, whereas expression of CD45RA and CD27 was often reduced as compared with naïve T cells. In patients with 0.2–0.5% MART-1-specific T cells, both naïve and antigen-experienced phenotypes were found.

In summary, in line with prior data<sup>(20)</sup>, two groups of patients can be distinguished: one group [15 (45%) of 33] with low numbers of MART-1-specific CD8<sup>+</sup> T cells in peripheral blood and a second group [18 (55%) of 33] with high numbers of MART-1-specific T cells. The phenotype of the MART-1-specific T cells of the majority of these patients has been analyzed; the analysis confirms that in patients with low numbers of MART-1-specific T cells, these cells display a naïve phenotype, whereas those cells in patients having high numbers are phenotypically antigen experienced.

#### *Melanoma-Specific T cells and Survival.*

Although the presence of melanocyte differentiation antigen-specific T cell immunity in patients with stage IV disease is well documented, the relevance of these T-cell responses in terms of response to treatment or survival remain unknown. We, therefore, attempted to relate the presence of melanoma-specific T cells to survival in this large cohort of HLA-A\*0201<sup>+</sup> stage IV melanoma patients.

Median follow-up was 46 months. At the time of analysis, 55 patients had died and the median survival of all patients was 7.8 months (Fig. 3 ; 95% confidence

interval, 6.0–9.7 months). This is in close agreement with the median survival observed in several randomized clinical trials using dimethyltriazeno-imidazole-carboxamide- or temozolomide-based treatment regimens for advanced-stage melanoma patients<sup>(23, 24)</sup>.

To test whether the presence of circulating melanoma-specific CD8<sup>+</sup> T cells was related to clinical outcome, survival of patients that had detectable numbers of circulating melanoma-specific T cells was compared with those without these cells (Fig. 4A). This analysis revealed that the presence of T cells specific for melanocyte lineage antigens is not associated with a survival benefit (log-rank,  $P = 0.354$ ). Survival curves of patients with either MART-1 or tyrosinase-specific T cells compared with patients without any melanoma-specific T cells, likewise, do not show any detectable survival advantage ( $P = 0.721$  and  $P = 0.346$  respectively; Fig. 4, B and C). To test whether a possible antitumor effect would manifest itself in patients with MART-1-specific T cells displaying an effector/memory phenotype, survival was also analyzed for this group ( $n = 8$ ; Fig. 4D). No statistically significant difference between survival of patients with effector/memory T cells and those without circulating T cells was observed (log-rank,  $P = 0.224$ ).

In summary, the presence of melanoma-specific T cell populations in peripheral blood does not lead to a survival benefit for patients with advanced-stage melanoma.

We considered the possibility that the melanoma-specific T cells we observed could have a limited capacity to kill tumor cells, for instance, because of a low avidity. In such a scenario, a high T-cell response would not predict a subsequent antitumor effect but would merely be a reflection of a growing tumor mass. To test this hypothesis, patients were divided into three groups: those with disease progression within 2 months after blood sampling, those with stable disease, and those with partial/complete response within this time period. When comparing these groups for the presence of circulating MART-1-specific T cells, we found a statistically significant (two-sided  $P = 0.0133$ , Cochran-Armitage trend test) predominance of high numbers ( $\geq 0.1\%$ ) MART-1 specific T cells in the group of patients with progressive disease in the first 2 months after blood sampling (Table 3)

#### *Immune Escape through Loss of HLA-A Expression.*

Tumors may evade the action of tumor-specific T cells by down-regulation of either MHC class I cell surface expression or by reducing antigen expression. To address this issue, we examined antigen and HLA-A expression by immunohistochemistry in tumor material that was available from only 17 of the 62 patients (data not shown). All 17 melanoma metastases expressed MART-1 and tyrosinase, whereas gp100 expression was homogeneously expressed in tumor material of 13 of 17 patients. Twelve of these 17 patients had circulating MART-1-specific T cells, 3 patients had tyrosinase-specific T cells, and 1 patient had gp100-specific T cells. In 7 of 17 metastases, HLA-A expression was lost. Lack of HLA-A expression was observed in 5 of 12 metastases from patients with detectable melanoma-specific T

cells, and 2 of 5 patients without detectable melanoma-specific T cells. These data demonstrate that although MHC class I loss does not directly correlate with the presence of detectable tumor-specific T-cell responses, the frequent occurrence of class I loss suffices to explain the lack of antitumor effect in a substantial fraction of the advanced-stage melanoma patients.

### **Discussion**

Several groups have reported the presence of naturally occurring melanoma-specific T-cell reactivity in melanoma patients (7, 9, 25, 26, 27, 28, 29, 30, 31), but little is known on the time frame of appearance and even less on the possible role of these cells in limiting tumor outgrowth. To address this issue, we have examined spontaneous cytotoxic T-cell immunity against melanosomal antigens, MART-1, tyrosinase, and gp100 in a large group of advanced-stage (IV), HLA-A\*0201-positive melanoma patients and have correlated these responses with patient survival. T-cell responses were analyzed before the initiation of treatment and should, therefore, reflect the natural response of the immune system to the growing tumor mass.

These data demonstrate that, in accordance with other studies, substantial expansions of CD8<sup>+</sup> T cells specific for melanosomal antigens can occur in patients with metastatic disease, amounting up to 18% of the total CD8<sup>+</sup> T-cell population in peripheral blood. Importantly, these high numbers of tumor-specific CD8<sup>+</sup> T cells do not have a measurable positive influence on patient survival.

Although the primary goal of this study was to evaluate a postulated benefit of spontaneous melanocyte lineage-specific T-cell responses, it may be useful to discuss these data in relation to immunotherapeutic approaches in this patient group. Several mechanisms may account for the fact that the T-cell populations specific for these melanoma antigens do not measurably impinge on tumor growth, and, depending on the mechanism involved, immunotherapeutic approaches that aim to strengthen these responses may or may not be worth pursuing. We considered two mechanisms: “immune escape” and “insufficient number or activity of tumor-specific T cells.

With regard to immune escape, tumors may evade the action of tumor-specific T cells by down-regulation of either MHC class I cell surface expression or by reducing antigen expression. In the tumor samples available for this study, we found loss of HLA-A expression in a substantial portion of the cases, whereas in all of the cases, antigen expression was maintained. This frequent occurrence of class I loss suffices to explain the lack of antitumor effect in a substantial fraction of the advanced-stage melanoma patients. In addition, it cannot be excluded that in part of the remaining cases, escape from T-cell attack is achieved by other means.

With regard to insufficient number or activity of tumor-specific T cells, in 7 of 12 patients with detectable tumor-specific T-cell reactivity, there was no concomitant loss of HLA-A or antigen expression. It is tempting to speculate that in these patients the number and/or activity (be it either homing capacity or cytolytic



function) of the tumor-specific T cells was insufficient to mediate a significant antitumor effect. Because vitiligo is associated with successful immunotherapy<sup>(1, 2, 3, 4)</sup>, further indirect evidence for this notion is provided by the observation that none of the 42 patients with detectable T-cell responses against melanosomal antigens showed signs of vitiligo. Lack of vitiligo despite substantial numbers of T cells in some of the patients may point to functional differences between T cells induced by immunotherapy and the spontaneous responses observed in this study.

MHC tetramers used in this study are indicative of the T-cell receptor specificity of the different melanoma-associated antigen-specific T-cell populations. It has been demonstrated that MHC tetramer binding does not always fully correlate with functionality<sup>(32)</sup>. However, in one patient the available blood samples did allow simultaneous MHC tetramer and intracellular IFN- $\gamma$  staining. In this patient with high numbers of both MART-1 and gp100-specific T cells, intracellular IFN- $\gamma$  was produced against peptide-pulsed target cells directly *ex vivo*. This result, however, does not preclude the possibility that for other patients the detected melanoma-specific T-cell populations were nonfunctional or tolerized.

On the basis of the current data, it seems that spontaneously occurring melanocyte lineage-specific immunity in stage IV melanoma patients is largely a reflection of increasing tumor mass and spreading, and stage IV melanoma patients are unlikely to benefit from these immune responses other than in exceptional cases. These melanoma-specific T cells may be functionally distinct from T cells that play a role in situations of spontaneous regression that rarely occur in these patients or from T cells that are detected on successful immunotherapy. Escape from immunity by loss of expression of target molecules either by immune selection or genetic instability of tumor cells forms one major factor that limits the effect of tumor-specific CTLs in part of this advanced-stage patient group. In the remaining patients, the limited efficacy of the tumor-specific T-cell response may well be related to the poor quality and suboptimal numbers of tumor-specific T cells at early time points during the disease. Efforts to enhance these responses by vaccination strategies or through adoptive therapy are, therefore, worth pursuing and are meeting increasing success<sup>(4, 33, 34)</sup>.

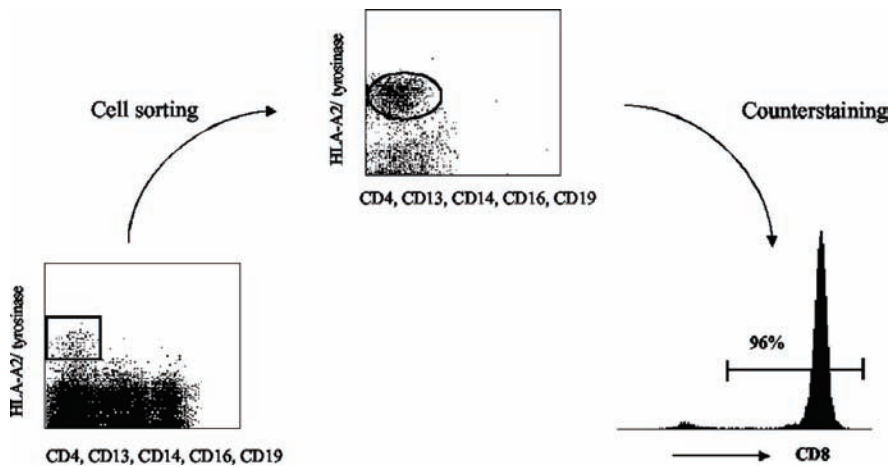
#### **Acknowledgements**

We thank Colette van den Bogaard and Willeke van den Kastele for their help with sample preparation, and Esther Kueter and Raquel Gomez for preparing MHC-tetramers.

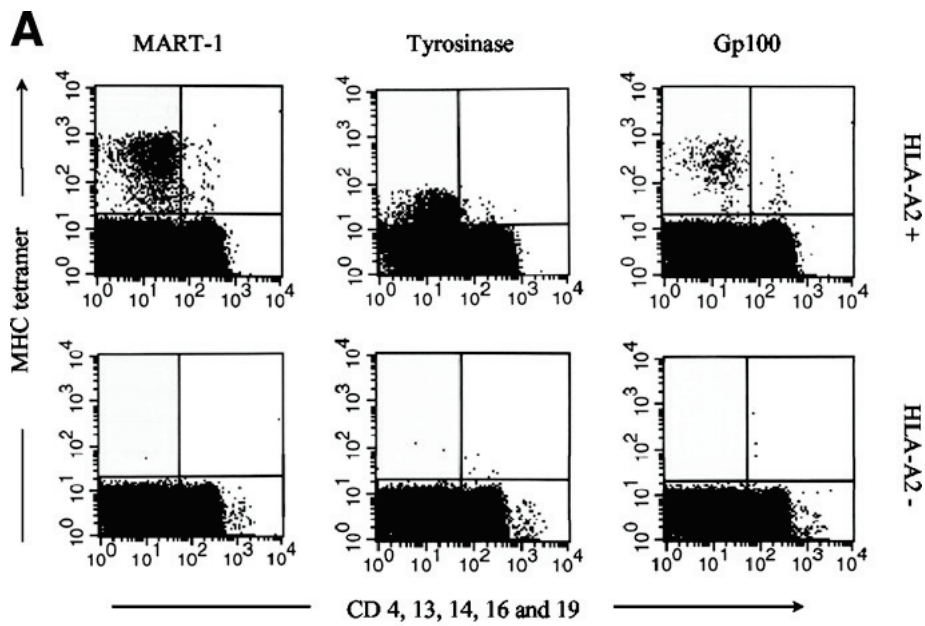
**Table 1** Patient characteristics

NO. OF PATIENTS	62
MEDIAN AGE	48 YR (RANGE, 21–79 YR)
SEX	
FEMALE	24 (39%)
MALE	38 (61%)
METASTATIC SITES, <i>N</i> (MEDIAN)	2 (1–5)
VISCERAL	42 (68%)
NONVISCERAL	20 (32%)
SERUM LACTATE DEHYDROGENASE (MEDIAN)	370
TREATMENT	
DTIC <sup>a</sup>	5 (5.1%)
TEMOZOLOMIDE	10 (6.2%)
CHEMOBIOTHERAPY	47 (76%)

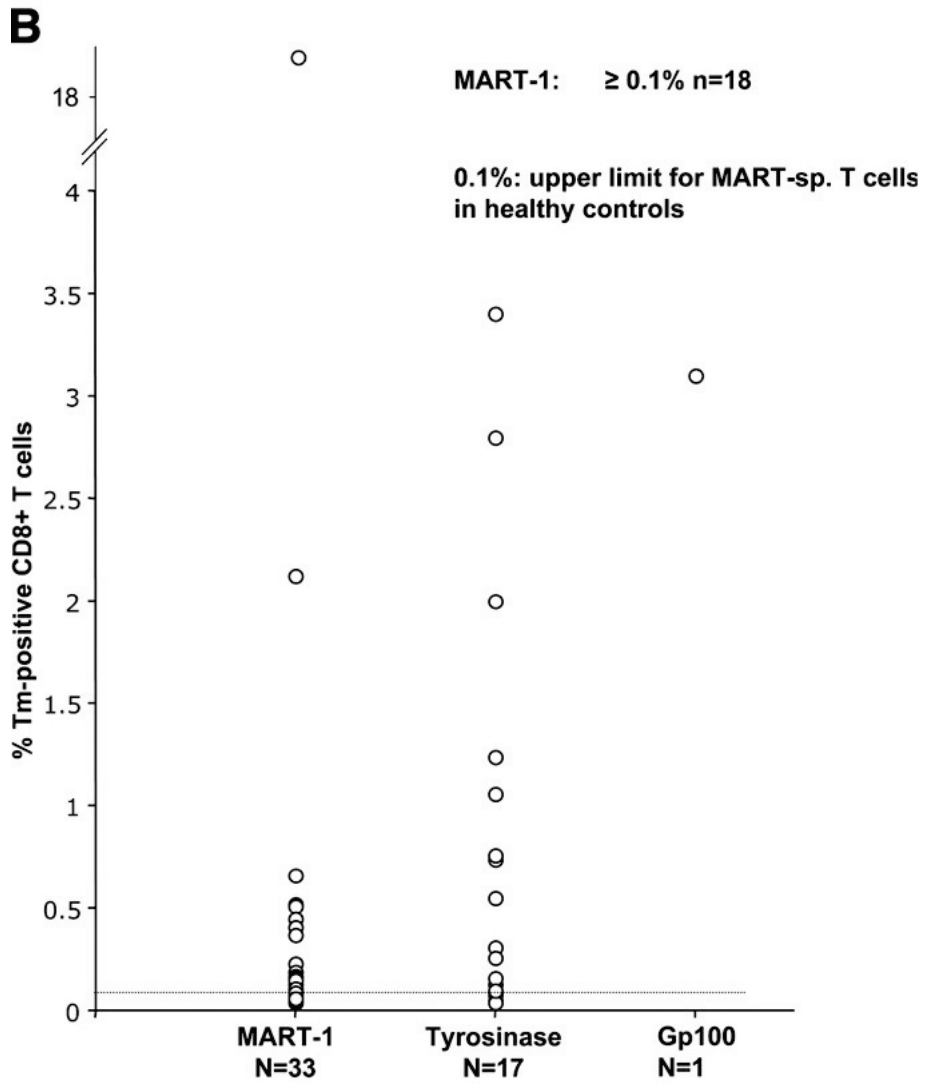
<sup>a</sup> DTIC, dimethyltriazeno imidazole carboxamide.



**Fig. 1.** Peripheral blood cells were stained with HLA-A2.1 tetramers containing the tyrosinase<sub>368–376</sub> peptide followed by staining with a panel of lineage antibodies, as described in “Patients and Methods.” Cells were analyzed by flow cytometry (left panel), and lineage antigen negative and MHC tetramer-positive cells were sorted by fluorescence-activated cell sorting (Cell sorting). Enriched MHC tetramer-positive, lineage antigens negative cells (middle panel) were briefly incubated at 37°C, stained with CD8 monoclonal antibody, and reanalyzed by flow cytometry (right panel). Typically >95% of these cells are CD8<sup>+</sup>.



**Fig. 2. A**, representative dot plots of MHC-tetramer staining versus lineage antibodies. Top three panels, HLA-A\*0201 melanoma patients (HLA-A2+) with detectable MART-1<sub>26-35 (27L)</sub><sup>-</sup>, tyrosinase<sub>368-376 (370D)</sub><sup>-</sup>, and gp100<sub>280-288 (288V)</sub><sup>-</sup>-specific CD8<sup>+</sup> T cells in peripheral blood. Bottom three panels, control staining of HLA-A2.1-negative melanoma patients (HLA-A2-) with HLA-A2.1/MART-1, tyrosinase, and gp100 tetramer.



B, scatter diagram of all patients with circulating melanoma-specific T cells, separated by antigen-specificity (MART-1, tyrosinase, or gp100). On the basis of the literature, presence of naïve MART-1-specific T cells (MART-sp. T cells) amounts to about 0.1%<sup>(20)</sup>. Tm-positive, MHC tetramer-positive.

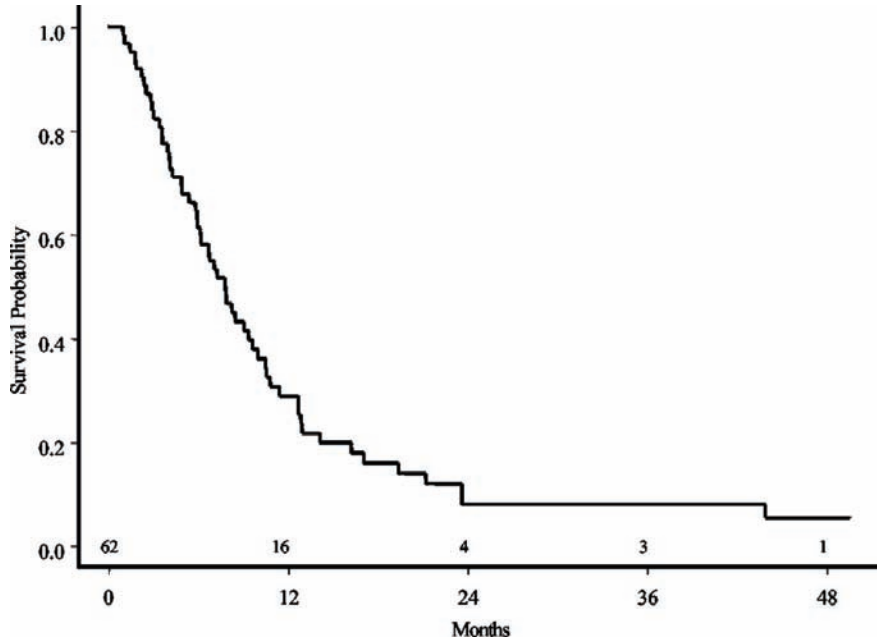


PATIENT NO.	CD45RA (%)	CD27 (%)	CD45RO (%)	CCR7 (%)	MART-1-SPEC. <sup>a</sup> T CELLS (%)
GROUP A					
NKI9822	92	93	17	NT	0.5
NKI9834	100	100	45	NT	0.2
NKI9829	85	80	15	NT	0.2
NKI9852	75	100	30	NT	0.2
NKI9839	65	83	28	NT	0.1
NKI9843	91	100	0	NT	0.1
NKI9818	75	100	13	NT	0.1
NKI0032	91	79	9	82	0.1
NKI0057	94	95	23	76	0.05
GROUP B					
NKI9835	34	64	76	NT	17.3
NKI9836	51	31	52	NT	2.8
NKI9828	81	26	51	NT	2.6
NKI9849	14	97	98	NT	0.9
NKI9810	30	38	87	NT	0.9
NKI0002	99	31	64	1	0.5
NKI0043	3	27	93	3	0.2
NKI0021	84	54	28	7	0.2

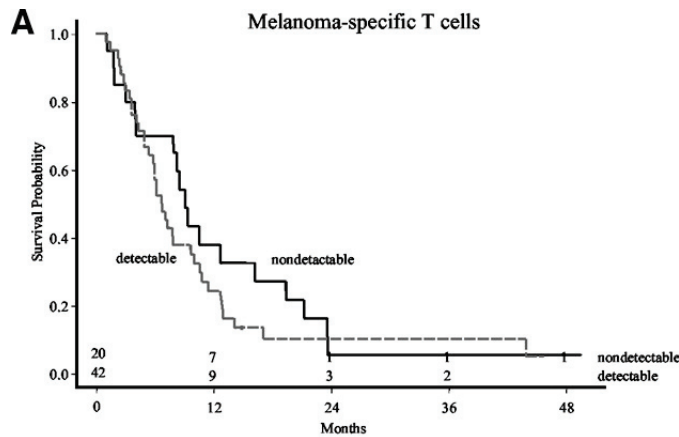
<sup>a</sup> MART-1-spec., MART-1-specific; nt, not tested.

**Table 2** Naïve versus effector/memory phenotype of MART-1-specific T cells.

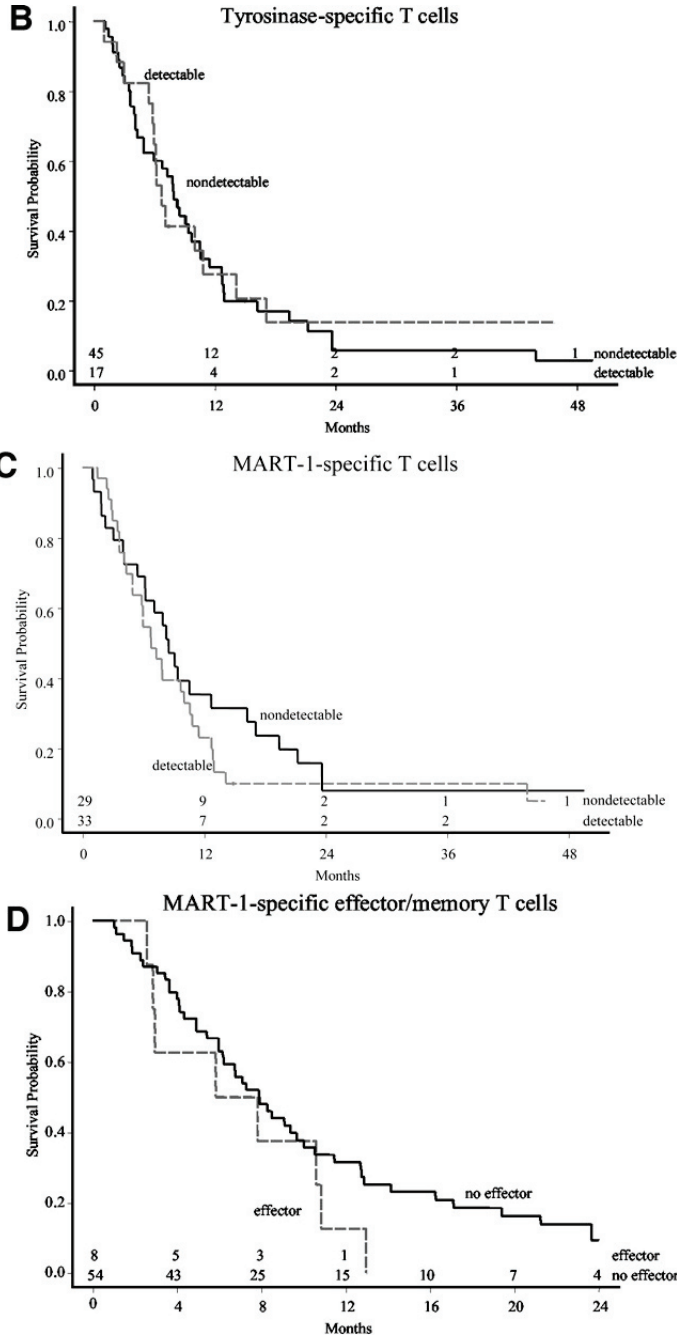
Panel antibody negative and MHC tetramer-positive T cells were costained with the indicated antibodies. Numbers represent percentages surface antigen positive cells from total MART-1-specific CD8<sup>+</sup> T cells. On the basis of differential expression of CD45RA, CD45RO, and CD27, patients with circulating MART-1-specific T cells can be divided into two statistically distinct groups, A and B. Group A, MART-1-specific T cells displaying a naïve phenotype with high CD45RA (Wilcoxon test, 2-sided P = 0.033), high CD27 (Wilcoxon test, 2-sided P = 0.0015), and low CD45RO (Wilcoxon test: 2-sided P = 0.0004). When tested, CCR7 expression was high, supporting a naïve phenotype. The frequency of these T cells in peripheral blood is low, mostly <0.2% of peripheral CD8<sup>+</sup> T-cell pool. Group B, MART-1-specific T cells present at higher frequency in peripheral blood (mostly <0.5% of CD8<sup>+</sup> T-cell pool) display an effector/memory phenotype with mostly high expression of CD45RO and low CCR7 expression (when tested).



**Fig. 3.** Kaplan-Meier survival curve of total cohort of HLA-A\*0201<sup>+</sup> stage IV melanoma patients. The median survival is 7.8 months (95% confidence interval, 6.0–9.7 months) with 55 deaths.



**Fig. 4.** Kaplan-Meier survival curves of HLA-A\*0201<sup>+</sup> patients with and without circulating melanoma-specific T cells. A, MART-1, tyrosinase- and gp100-specific CD8<sup>+</sup> T cells; log-rank P = 0.354; hazard ratio, 1.32 [95% confidence interval (CI), 0.74–2.33].



B, tyrosinase-specific T cells only; log-rank  $P = 0.721$ ; hazard ratio, 0.89 (95% CI, 0.48–1.63). C, all MART-1-specific T cells; log-rank  $P = 0.346$ ; hazard ratio, 1.29 (95% CI, 0.75–2.21). D, established effector/memory MART-1-specific T cells ( $n = 8$ ); log-rank  $P = 0.224$ .

**Table 3** Expanded MART-1-specific T cells preferentially in patients with rapid progressive disease<sup>a</sup>. Patients were divided into three groups depending on their evaluated response to two courses of treatment. Treatment consisted of chemotherapy ± biotherapy (see Table 1 ). Responses were evaluated according to RECIST<sup>b</sup> criteria<sup>(35)</sup>. The presence of expanded (>0.1%) MART-1-specific cells was compared with the presence of no or few MART-1-specific T cells (<0.1%).

RESPONSE	MART-1-SPECIFIC CD8+ T CELLS		TOTAL
	<0.1%	>0.1%	
CR/PR	12	2	14
SD	11	0	11
PD	21	16	37
TOTAL	44	18	62

<sup>a</sup> Statistical analysis was performed with Cochran-Armitage trend test.

<sup>b</sup> RECIST, response evaluation criteria in solid tumors; CR/PR, complete or partial response; SD, stable disease; PD, progressive disease.



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