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Cytotoxic T cell responses to melanocyte antigens: Vitiligo as the effective counterpart of melanoma immunity

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Abstract

By the use of HLA/epitope tetramers, a tool to measure the frequency of specific CTL precursors independently of their functional state, our group demonstrated that melanocyte-specific CTLs are present in the peripheral blood of both melanoma and vitiligo patients. Notably, however, vitiligo cells were capable of lysing HLA-matched melanoma cell lines much more efficiently than CTLs obtained from melanoma patients. The increased anti-tumor activity of vitiligo CTLs was mainly due to their superior TCR affinity. The possibility that our results might pose the

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in vitro research basis to the development of new therapies for melanoma, such as the genetic transfer of high affinity anti-tumor TCRs from vitiligo donors to HLA-matched melanoma patients, is discussed.

Introduction

Significant progress in the understanding of the immune biology of melanoma has evolved from the identification of melanoma antigens recognized by T cells [1-4]. However, although the presence of melanoma-specific CTLs in cancer patients demonstrate that tumor cells may not completely evade immune recognition, the patient's immune system can only rarely counteract tumor growth [5-7]. Vitiligo is a relatively frequent skin disease characterized by the presence of depigmented macules and patches as a consequence of local melanocyte loss [8]. An unusual facet of vitiligo is its relation to melanoma. CTLs directed to self antigens shared by normal melanocytes and melanoma cells are found in both conditions and imply a breakdown of tolerance [9-14], yet the resulting immune reaction is the opposite [reviewed in 15]. In this respect, reactivity to vitiligo melanocytes may be regarded as the effective variant of an immune response often ineffective in melanoma. These data, together with the resistance of melanoma to conventional chemotherapeutic and radiotherapeutic approaches, have made the melanoma/vitiligo dichotomy an important model for immunologic investigation.

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 yet the absence of protective immune responses to tumors. Being the cytotoxic response a key component of both tumor immunity and autoimmunity, a comparison between anti-melanocytic CTLs from vitiligo and melanoma patients can answer the question as to whether differences in CTL precursor frequency, their status of activation or their affinity for the peptide-MHC complex have a role in determining these opposite immune reactions.

By the use of HLA/epitope tetramers, a tool to measure the frequency of specific CTL precursors independently of their functional state [16], our group demonstrated that melanocyte-specific CTLs are present in the peripheral blood of both primary melanoma and vitiligo patients [17-20]. Notably, however, vitiligo cells are capable of lysing HLA-matched melanoma cell lines much more efficiently than CTLs obtained from melanoma patients [17-19]. The increased anti-tumor activity of vitiligo CTLs is mainly due to their superior TCR affinity.

Our results might pose the *in vitro* research basis to the development of new therapies for melanoma, such as the genetic transfer of high affinity anti-tumor TCRs from vitiligo cells to melanoma peripheral lymphocytes with the

aim to redirect their antigen specificity toward a target relevant to anti-tumor immunity.

High frequencies of circulating melanocyte-specific CD8$^+$ T lymphocytes are found in both melanoma and vitiligo patients

Our first aim was to obtain an accurate estimate of the precursor frequencies of CTLs specific for three different melanocyte differentiation antigens in melanoma and vitiligo patients. We thus synthesized three fluorescent HLA-A2 tetramers complexed with both parental and modified melanoma peptides from Melan-A/MART1 (peptide A27L), tyrosinase (peptide 1-9) and gp100 (peptide 209M). The specificity of each tetramer had already been confirmed in our laboratory by staining CTL lines or clones specific for HLA-A2 in association with the peptide of interest. The limit of detection of each tetramer, determined by titrating known antigen-specific T cells into normal PBMC, was established to be 0.04% of CD8$^+$ T cells for A2/Melan-A and A2/tyrosinase tetramers, and 0.06% of CD8$^+$ T cells for A2/gp100. We used these three tetramers to stain PBMC from sixteen HLA-A2+ melanoma patients and twelve HLA-A2+ vitiligo patients directly *ex vivo*, without any antigen-specific stimulation [17, 18 and unpublished results]. Clinical characteristics of the patients are presented in **Table I**. High numbers of both Melan-A/MART1- and tyrosinase-specific cells were detected in the majority of patients (**Table I and Fig.1**). Detection was dependent on the expression of HLA-A*0201, as none of several A*0201-negative melanoma and vitiligo patients had detectable tetramer$^+$ cells *ex vivo* [17, 18]. The frequency of A2/Melan-A tetramer$^+$ cells varied between 0.02% and 0.35% (mean 0.11%) of the total CD8$^+$ population in melanoma and between 0.07% and 0.28% (mean 0.14%) of the total CD8$^+$ population in vitiligo; the frequency of A2/tyrosinase-positive cells between 0.02% and 0.24% (mean 0.13%) in melanoma and between 0.10 and 0.22 (mean 0.16%) in vitiligo (**Table I** and **Fig. 1**). On the contrary, very few gp100-specific CD8$^+$ T cells could be observed *ex vivo* by tetramer staining (**Table I**), and we demonstrated this was due to a low precursor frequency of these cells in both groups of patients [17].

These data demonstrated the presence of comparably high frequencies of melanocyte-specific CTLs in the peripheral blood of both melanoma and vitiligo patients.

As a second aim, we assessed whether the melanocyte-specific CTLs found in the two diseases had a history of antigen exposure and if they were capable of exerting intact cytotoxic activities. The activation status of these cells was expressed in terms of naive/effector/memory component. We observed that all patients contained variable proportions of A2/Melan-A tetramer$^+$ lymphocytes displaying an effector/memory phenotype and that their

Table 1.

				A2-tetramer $^{+}$ cells ^{b}		
Melanoma Patient	Sex ^{a}	Stage		Melan-A	Tyrosinase	gp100
ALO	F	IV ^{c}		**0.05**	_	_
API	M	IIA/T2aN0M0		**0.11**	**0.07**	0.01
ATO	M	IIA/T3N0M0		**0.17**	_	_
BBR	M	IIA/T2aN0M0		**0.11**	_	_
EBI	F	I/T1aN0M0		**0.35**	_	0.00
CPA	F	IIA/T3aN0M0		**0.15**	_	_
GCO	F	II/T2bN0M0		**0.10**	_	0.00
GGE	M	I/T1aN0M0		**0.04**	_	_
GPE	M	IIA/T3N0M0		0.02	0.02	0.01
IFE	M	IB/T2N0M0		0.02	**0.05**	0.02
LCO	F	I/T1aN0M0		**0.08**	_	0.00
OBO	M	IIA/T3N0M0		**0.16**	**0.18**	0.00
SCO	F	IB/T2N0M0		**0.10**	**0.23**	0.04
SDB	F	IIA/T3N0M0		**0.22**	**0.24**	0.01
SLA	M	IIB/T4N0M0		**0.10**	**0.10**	0.01
TBE	M	I/T1aN0M0		**0.05**	_	_
Vitiligo Patient		Disease Extension (%)	Course ^{d}			
AAN	F	Hands, arms (5%)	Active	**0.12**	_	0.00
ADL	M	Diffuse (80%)	Stable	**0.07**	**0.14**	0.02
CLA	F	Arms (18%)	Stable	**0.07**	**0.14**	0.04
LPA	M	Face, arms, legs (45%)	Active	**0.21**	**0.19**	0.01
MBO	F	Hands, face (18%)	Stable	**0.28**	**0.21**	0.04
PSA	F	Diffuse (80%)	Stable	**0.19**	**0.20**	0.04
RNA	F	Diffuse (80%)	Stable	**0.09**	**0.13**	**0.06**
RSA	M	Hands, penis (13%)	Stable	**0.07**	**0.10**	0.01
SCA	M	Face, penis, hands, arms, legs (30%)	Active	**0.17**	_	0.01
SCH	F	Hands, face (1%)	Stable	**0.11**	_	0.00
SRI	M	Diffuse (80%)	Active	**0.09**	**0.12**	_
TSC	F	Abdomen, feet (18%)	Stable	**0.22**	**0.22**	0.00

[a] M, male; F, female.

[b] Percentage of tetramer $^{+}$ cells relative to total CD8 $^{+}$ T cells.

[c] Spontaneous remission.

[d] The disease course was defined as active vitiligo (newly depigmented lesions during the last three months) or stable vitiligo (no recent depigmented or repigmented lesions in the three months prior to examination).

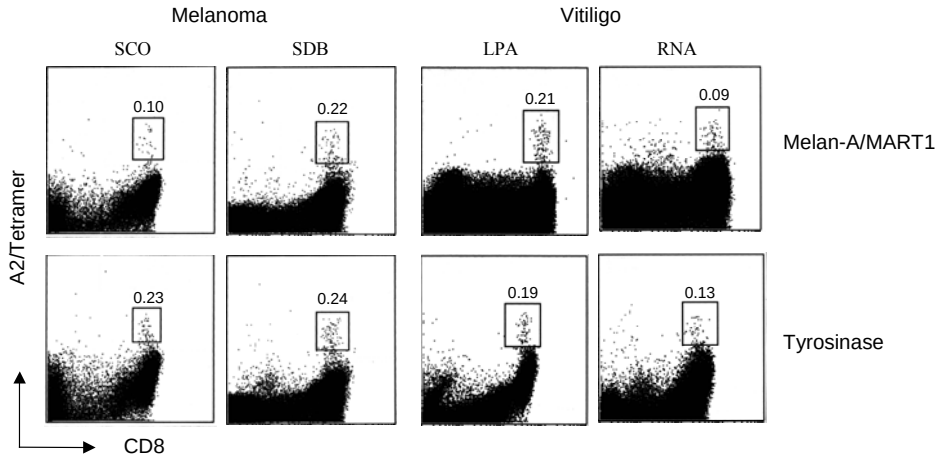


Figure 1. Detection of circulating melanocyte-specific CD8$^+$ T Lymphocytes in melanoma and vitiligo patients. Stainings with two different peptide-loaded HLA-A*0201 tetramers are shown on two melanoma and two vitiligo patients. Cells were gated by forward- and side-scatter for lymphocytes. Gated populations are plotted as CD8-staining (horizontal axis) versus tetramer-staining (vertical axis). Boxed populations, CD8$^+$ tetramer$^+$ cells (frequencies within total CD8$^+$ T cells above each box). Upper side, patient identifiers.

number was comparable in melanoma and vitiligo patients (mean 25.7% in melanoma versus 23.9% in vitiligo, difference not significant; unpublished results). The lytic capacity of tetramer$^+$ cells was analyzed by complementing the use of soluble HLA/peptide complexes with intracellular FACS analysis; in particular, we assessed if melanocyte-specific cells had intact cytotoxic properties through perforin release analyses. The results indicated that comparable numbers of tetramer$^+$ cells contained perforin (mean 16% in melanoma versus 17% in vitiligo, difference not significant; unpublished results).

These observations were in agreement with previous literature data indicating that Melan-A-specific CTLs from both melanoma and vitiligo patients frequently show signs of an *in vivo* antigen encounter [17, 18, 21, 22] and indicate that the activation status of melanocyte-specific CTLs is not a distinctive feature of either diseases.

Melan-A/MART1-specific T cells from vitiligo patients are characterized from high avidity and tumor-reactivity

Avidity of antigen recognition is an important feature of tumor-specific T lymphocytes, determining their capability to kill tumor cells. We measured this important feature as the ability of the melanocyte-specific cells to respond to the stimulation provided by titrated peptide-MHC complexes. As the T cell response is a peptide concentration-dependent sigmoidal function, the peptide

concentration that elicits a half-maximal response can be considered a measure of the functional cell avidity. We assessed the functional avidity of A2/Melan-A tetramer^{+} T-cell clones generated from peptide-sensithyzed T cell lines from two melanoma (SDB and OBO) and one vitiligo (PSA) patients. Titration curves over a wide concentration range (5000 nM to 0.03 nM) of the Melan-A analog peptide A27L [23] were generated and clone avidity was defined as the concentration of peptide required to obtain half maximal lysis (nM[50%]) at an E:T cell ratio of 5:1 in a standard cytotoxicity assay. In the case of melanoma, all clones could specifically lyse peptide pulsed target cells, but they demonstrated a broad range of avidity, varying between 0.4 and 113 nM (the coefficient of variation is 215.2) [18]. Similarly, Melan-A/MART1-specific CTL clones were established from the vitiligo patient 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 [17].

Next, we examined whether the melanocyte-specific CTLs isolated from both melanoma and vitiligo are capable of exerting tumor reactivity. We assessed the lytic activity of CTL clones against HLA-matched melanoma cell lines, which present the peptide through endogenous processing pathways. We found that tumor reactivity correlated with clone avidity, as only those clones exhibiting very high avidity of antigen recognition were capable of efficiently killing tumor cells (**Fig. 2** for some representative cases) [17, 18].

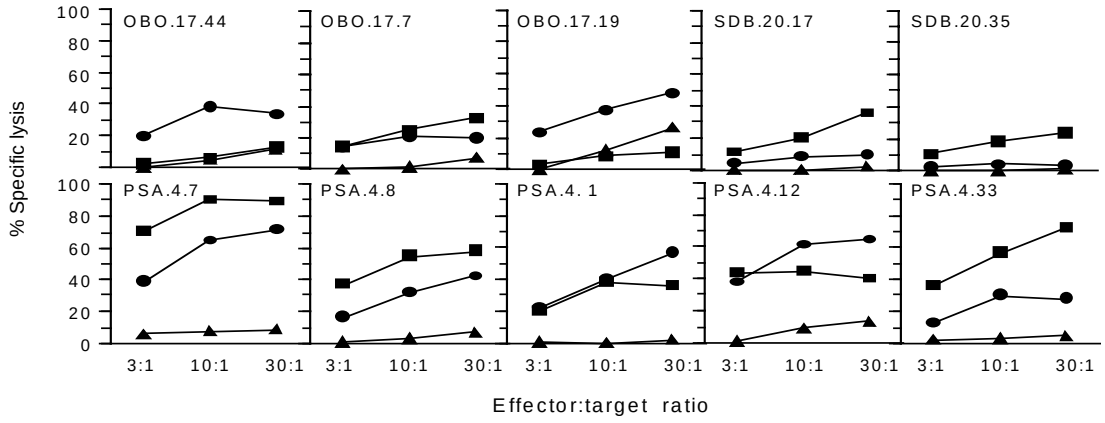


Figure 2. Tumor-reactivity of Melan-A/MART1-specific CTL clones. T cell clones, derived by limiting dilution from Melan-A/MART1-sensityzed cell lines of melanoma patients OBO and SDB and of vitiligo patient PSA, were tested in standard 4-h chromium release assays for their ability to lyse two different, HLA-matched, melanoma cell lines (501 mel and 213 mel). Lysis of T2 cells pulsed with a HLA-A*0201 restricted, irrelevant peptide (gp100-209M) was performed as control. Points are means of triplicates. Shown is one of several experiments done. ●, 501 mel; ■, 213 mel; ▲, T2 + irrelevant peptide.

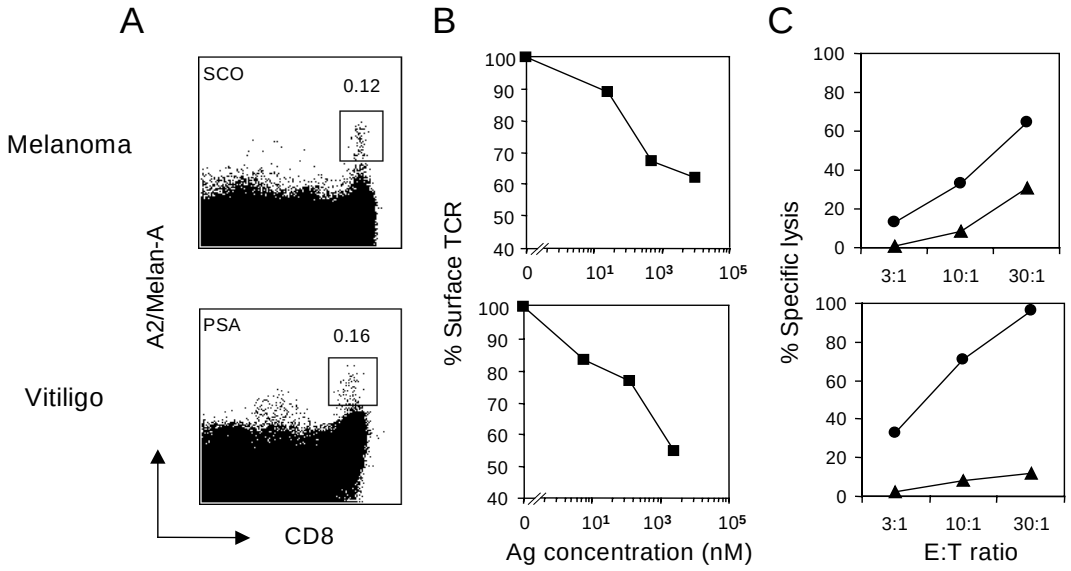


Figure 3. Functional avidity and tumor-reactivity of *ex-vivo* sorted Melan-A-specific T cells. (A) Freshly isolated PBMC from one HLA-A2 melanoma patient (SCO) and one HLA-A2 vitiligo patient (PSA) were stained with A2/A27L tetramers and anti-CD8 mAb and analyzed by flow cytometry. The percentage of tetramer$^+$ cells within the CD8$^+$ population is shown. Cells within the gate were FACS sorted using a FACSVantage SE. (B) Sorted Melan-A-specific CD8$^+$ T cells, that had been shortly expanded in vitro, were tested for their peptide specificity through TCR down-regulation experiments. HLA-A2$^+$ TAP deficient T2 cells were pulsed with various concentrations of Melan-A A27L peptide and added to effector cells at a final E:T ratio of 1:5. After 3 h at 37°C, TCR internalization was measured by flow cytometry using A2/Melan-A tetramers. The percentage of surface TCR was calculated with a modified method [see ref. 19]. (C) Sorted tetramer$^+$ cells, that had been expanded in vitro, were assayed for specific lysis against a Melan-A-expressing, HLA-matched melanoma cell line (501 mel) in a standard 4-h chromium release assay. T2 cells pulsed with a HLA-A2 restricted, irrelevant peptide (MAGE-3$_{271-279}$) were used as negative control. ●, 501 mel; ▲, T2 + irrelevant peptide.

To perform a direct comparison of whole melanocyte-specific T cell populations in the two diseases, freshly isolated PBMC from one melanoma (SCO) and one vitiligo (PSA) HLA-A2$^+$ patients were stained with HLA-A2 tetramers synthesized around the Melan-A A27L analog peptide [23] together with an anti-CD8 mAb and isolated by flow cytometry cell sorting [19]. As shown in **Fig. 3A**, the A2/Melan-A tetramer$^+$ T cells constituted 0.12% and 0.16% of the CD8$^+$ cells, respectively. Sorted cells were analyzed through peptide-specific TCR-down-regulation experiments and cytotoxicity assays against a HLA-matched melanoma cell line (**Fig. 3, B and C**). Both experiments confirmed the superior avidity and anti-tumor reactivity of Melan-A-specific T cells from vitiligo.

Overall, these data suggested that Melan-A/MART1-specific CTLs isolated from vitiligo patients possessed a higher avidity and exerted a superior anti-tumor activity than those from melanoma.

The increased anti-tumor activity of vitiligo CTLs is mainly due to their superior TCR affinity

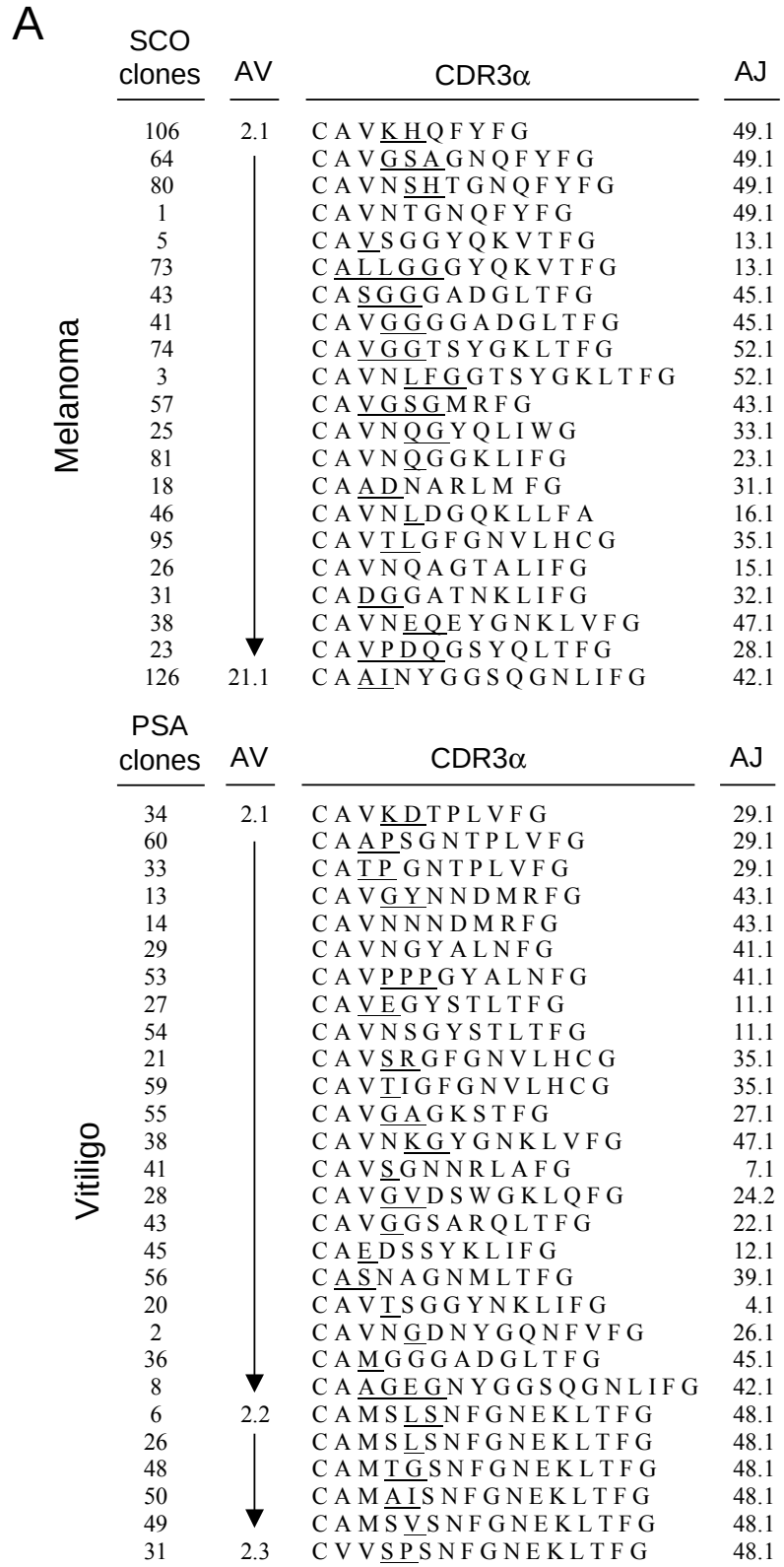
TCR downregulation occurs when T cells recognize specific antigen, i.e., peptides bound to major histocompatibility complex molecules expressed on antigen presenting cells (APCs) [24, 25]. In specific T-APC conjugates TCRs are downregulated in an antigen dose- and time-dependent fashion [25] and the extent of TCR downregulation (as measured by decreased cell surface staining for TCR) is dependent on the concentration and quality of the agonist displayed on the APC surface, but is not influenced by costimulatory molecules [25-27]. In this respect, the TCR downregulation assay is a good indicator of TCR affinity. Recently, we used a tetramer-based TCR downregulation assay developed by ourselves to measure the degree of peptide-induced response in the whole Melan-A-specific cell fractions of melanoma and vitiligo patients [manuscript submitted]. We found a higher affinity of T cell receptor in vitiligo cells, as the concentration of peptide required for 50% of maximal downregulation was 6-fold lower than in melanoma.

These results demonstrated that the augmented functional avidity of vitiligo CTLs, which results in their increased anti-tumor activity, was mainly due to a higher affinity of their T cell receptors.

Strong TCR-α chain conservation in Melan-A-specific cells

TCR-α and -β chains are composed of somatically rearranged V, D and J germline-encoded gene segments that confer antigen specificity [28]. Recent crystallographic analyses revealed that TCR-α has more contacts with peptide than TCR-β, suggesting the possibility that peptide recognition predominantly relies on TCR-α [29-32]. T cells specific for the self antigen Melan-A/MART1 [33, 34] possess an exceptionally high precursor frequency in human histocompatibility leukocyte antigen-A2 individuals [35]. This provided a unique situation for assessment of the structural relationship between TCR and peptide/MHC ligand at both the pre- and post-immune levels. Freshly isolated PBMC from one HLA-A2 vitiligo (PSA) and one melanoma patient (SCO) were stained with HLA-A2 tetramers synthesized around the Melan-A A27L peptide [23] together with an anti-CD8 mAb and analyzed by flow cytometry [19]. The $CD8^+$ $tetramer^+$ population was isolated by flow cytometry cell sorting and cloned by limiting dilution. To analyze the TCR repertoire displayed by the $CD8^+$ A2/Melan-A $tetramer^+$ T cells, RNA was extracted

Figure 4


A
Melanoma
SCO clones
AV
CDR3α
AJ
106 2.1 C A V K H Q F Y F G 49.1
64 C A V G S A G N Q F Y F G 49.1
80 C A V N S H T G N Q F Y F G 49.1
1 C A V N T G N Q F Y F G 49.1
5 C A V S G G Y Q K V T F G 13.1
73 C A L L G G G Y Q K V T F G 13.1
43 C A S G G G A D G L T F G 45.1
41 C A V G G G G A D G L T F G 45.1
74 C A V G G T S Y G K L T F G 52.1
3 C A V N L F G G T S Y G K L T F G 52.1
57 C A V G S G M R F G 43.1
25 C A V N Q G Y Q L I W G 33.1
81 C A V N Q G G K L I F G 23.1
18 C A A D N A R L M F G 31.1
46 C A V N L D G Q K L L F A 16.1
95 C A V T L G F G N V L H C G 35.1
26 C A V N Q A G T A L I F G 15.1
31 C A D G G A T N K L I F G 32.1
38 C A V N E Q E Y G N K L V F G 47.1
23 C A V P D Q G S Y Q L T F G 28.1
126 21.1 C A A I N Y G G S Q G N L I F G 42.1
Vitiligo
PSA clones
AV
CDR3α
AJ
34 2.1 C A V K D T P L V F G 29.1
60 C A A P S G N T P L V F G 29.1
33 C A T P G N T P L V F G 29.1
13 C A V G Y N N D M R F G 43.1
14 C A V N N N D M R F G 43.1
29 C A V N G Y A L N F G 41.1
53 C A V P P P G Y A L N F G 41.1
27 C A V E G Y S T L T F G 11.1
54 C A V N S G Y S T L T F G 11.1
21 C A V S R G F G N V L H C G 35.1
59 C A V T I G F G N V L H C G 35.1
55 C A V G A G K S T F G 27.1
38 C A V N K G Y G N K L V F G 47.1
41 C A V S G N N R L A F G 7.1
28 C A V G V D S W G K L Q F G 24.2
43 C A V G G S A R Q L T F G 22.1
45 C A E D S S Y K L I F G 12.1
56 C A S N A G N M L T F G 39.1
20 C A V T S G G Y N K L I F G 4.1
2 C A V N G D N Y G Q N F V F G 26.1
36 C A M G G G A D G L T F G 45.1
8 C A A G E G N Y G G S Q G N L I F G 42.1
6 2.2 C A M S L S N F G N E K L T F G 48.1
26 C A M S L S N F G N E K L T F G 48.1
48 C A M T G S N F G N E K L T F G 48.1
50 C A M A I S N F G N E K L T F G 48.1
49 C A M S V S N F G N E K L T F G 48.1
31 2.3 C V V S P S N F G N E K L T F G 48.1

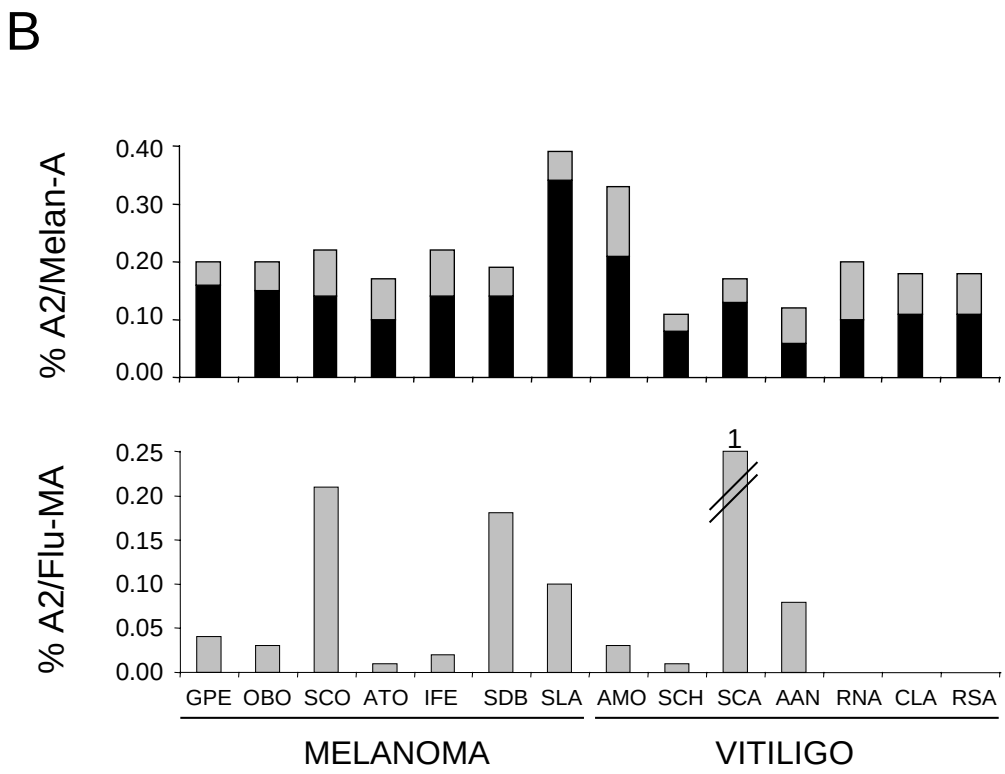


Figure 4. Dominant usage of TCRAV2 is a conserved feature of the Melan-A-specific peripheral repertoire. (A) TCR-α chain amino acid sequences of Melan-A-specific cell clones from one melanoma and one vitiligo HLA-A2 patients. CDR3α junctional amino acid sequences of *ex-vivo* sorted Melan-A-specific T cell clones are shown. Amino acids either partially or completely formed by non-template added nucleotides (N nucleotides) are underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession no. AY135832-AY135859. (B). PBMC from 7 HLA-A2 vitiligo patients and 7 HLA-A2 melanoma patients were enriched for $CD8^+$ cells with magnetic beads and analyzed by double staining with A2/Melan-A tetramers and anti-AV2.1 mAb. Bars represent the total percentages of $tetramer^+$ cells within the $CD8^+$ population; black parts represent the percentages of $AV2.1^+$ cells and gray parts the percentages of $AV2.1^-$ cells within the $tetramer^+$ $CD8^+$ population. Lower panel shows the same samples analyzed by double staining with A2/Flu-MA tetramers and anti-AV2.1 mAb.

from 30 vitiligo and 21 melanoma clones and cDNA subjected to RT-PCR using a panel of AV and BV oligonucleotides covering virtually 100% of the TCR-αβ repertoire. In line with previously published data concerning the heterogeneity of the Melan-A-specific TCR-β repertoire [35, 36], Melan-A-specific $CD8^+$ T cells from these patients were found to rearrange many distinct BV gene segments [19]. By contrast, all clones except one expressed the same rearranged AV gene segment (AV2, 28/28 clones; the AV rearrangement of two clones could not be determined) (**Fig. 4A**). Direct sequencing with an internal AC-specific primer was performed to determine

the complete TCR-α CDR3 region of the Melan-A-specific clones. The AV2 segment was always productively rearranged and the CDR3 regions were all different, indicative of a large degree of polyclonality in the specific TCR repertoire (**Fig. 4A**). To assess whether the selective use of this AV segment was a generalized feature of Melan-A-specific cells, PBMC from 14 HLA-A2 patients (7 with vitiligo and 7 with melanoma) were analyzed [19]. PBMC were enriched in $CD8^+$ cells with magnetic beads and stained with A2/Melan-A tetramers in association with an anti-AV2.1 mAb. The high percentages of AV2.1-expressing cells in the $tetramer^+$ population of all our patients (range 50-87%; mean ± SD, 67% ± 10.9%) was consistent with a highly preferential use of AV2 in forming the Melan-A-specific TCR (**Fig. 4B**). HLA-A2 tetramers built around the Flu-MA peptide were also used in conjunction with the AV2.1 mAb to stain these samples. None of the A2/Flu-MA $tetramer^+$ cells turned out to be $AV2.1^+$, indicating that preferential usage of this AV segment is specifically relevant for recognition of the self antigen Melan-A (**Fig. 4B**). This molecular analysis of many different Melan-A-specific T cell populations revealed that a structural constraint is imposed on the TCR for engagement with Melan-A peptides presented by HLA-A2.

In the same work [19], the origin of the $AV2^+$, Melan-A-specific T cells was directly investigated by tetramer staining T cells from the human thymus. Examination of CD8 single-positive thymocytes indicated that this preferential use in forming the Melan-A-specific TCR was mainly imposed by intrathymic positive selection.

Overall, these data demonstrated a dominant function of TCRAV2 segment in forming the TCR repertoire specific for the human self antigen Melan-A/MART1 and supported the view that antigen recognition is mediated predominantly by TCR-α.

The Melan-A-specific peripheral T cell repertoire comprises three groups of cells with variable avidity of Ag recognition

PSA is a female HLA-A*0201 homozygote patient affected by non-familial, nonsegmental, patchy vitiligo diffuse on the face, arms, hands, thorax, groin, axillae and legs. The disease affects 80% of the patient's body surface and it has been stable for more than ten years. The patient does not suffer from any other related autoimmune disease and remained free of therapy during the follow-up period relative to our study (three years). Freshly isolated PBMC from this patient were stained with A2/Melan-A tetramers and anti-CD8 mAb directly *ex vivo*, without any Ag-specific stimulation and analyzed by flow cytometry [20]. The A2/Melan-A $tetramer^+$ T cells represented 0.16% of the $CD8^+$ cells at this time point and exhibited a

mixed phenotype composed of naive ($CD45RA^+CD28^+$, 57%), effector ($CD45RA^+CD28^-$, 15%) and memory cells ($CD45RA^-CD45RO^+$, 24%) (data not shown). $CD8^+$ $tetramer^+$ population was isolated by flow cytometry cell sorting and sorted cells were immediately cloned by limiting dilution. 29 clones, all staining positive to the tetramers (not shown), were functionally characterized. Avidity of Ag recognition was assessed by titrating both Melan-A A27L analogue and Melan-A_{26-35} natural peptides in a functional CTL assay on T2 target cells. Three groups of clones were distinguishable according to their functional activity: group 1) clones that specifically lysed both Melan-A A27L- and Melan-A_{26-35}-pulsed T2 cells, group 2) clones that recognized Melan-A A27L peptide but failed to specifically lyse Melan-A_{26-35}-pulsed T2 cells, and group 3) clones that failed to significantly recognize both Melan-A peptides (**Fig. 5**). Redirected lysis experiments were performed to ascertain functionality of the clones belonging to group 3. Only one clone was found to be non-functional (not shown). TCR down-regulation upon peptide encounter was found to perfectly correlate with the functional avidity of the clones (**Fig. 5**), supporting the above classification.

In that paper [20] we demonstrated, for the first time, that many high avidity T cell clones from this patient were capable of specifically lysing normal, HLA-matched melanocytes and that many autoreactive clones persisted for more than three years in the patient's peripheral blood. These data, together with the skin-homing potential of the clones, directly pointed to the *in vivo* pathogenic role of melanocyte-specific CTL in vitiligo.

At variance with the T cell clones we previously isolated from a Melan-A-sensitized cell line from this vitiligo patient, which homogeneously exhibited high avidity of Melan-A recognition [17], *ex-vivo* isolated $tetramer^+$ T cell clones displayed a large heterogeneity in terms of both avidity and fine specificity. Clones reacting with the natural Melan-A_{26-35} peptide represented only a minority of the total population and were among the ones displaying higher avidity of recognition of Melan-A A27L analogue-pulsed cells. Surprisingly, a significant proportion of *ex vivo* sorted $tetramer^+$ T cell clones failed to recognize both Melan-A peptides. Possibly, these clones exhibited some degree of specificity for Melan-A Ag, sufficient to bind A2/Melan-A tetramers, but their functional avidity was too low to be detected in a functional assay. Though we cannot formally demonstrate it, we believe that these clones represent essentially naive cells that are specific for some of the many Melan-A epitope mimics described so far [37, 38] and that they do not participate to the anti-melanocyte immune response in this patient. This would explain the presence of a conspicuous fraction of naive A2/Melan-A $tetramer^+$ cells even in patients with a long-lasting disease.

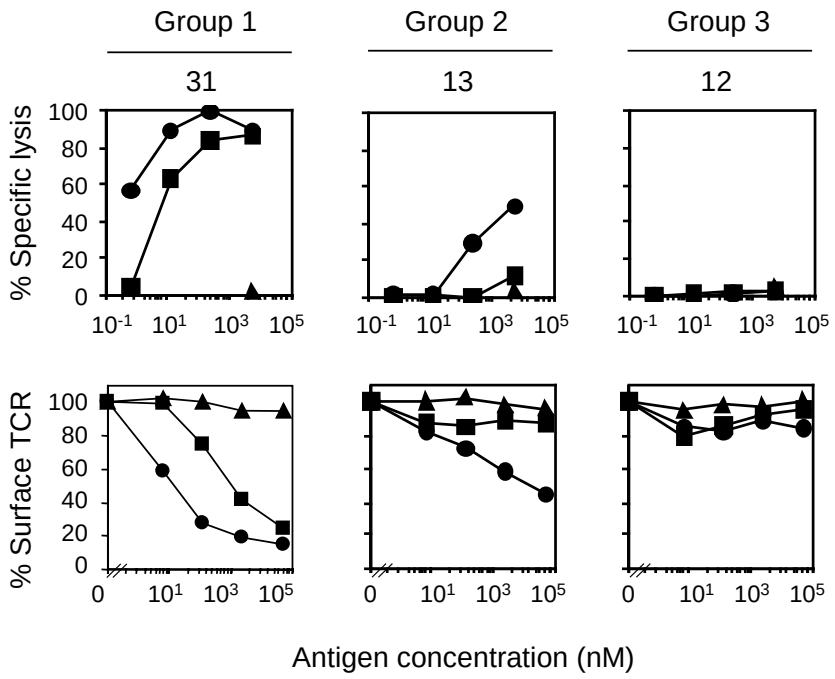


Figure 5. *Ex-vivo* sorted A2/Melan-A tetramer$^+$ T cells from a HLA-A2$^+$ vitiligo patient can be classified into three functional groups. Sorted A2/Melan-A tetramer$^+$ CD8$^+$ T cells were cloned by limiting dilution immediately after sorting and 29 independent clones were analyzed. Upper panel, avidity of three representative tetramer$^+$ vitiligo clones as measured in a standard 4 h chromium release assay against T2 cells pulsed with increasing concentrations of either the natural Melan-A_{26-35} (■) or the analogue Melan-A A27L (●) peptide, at an effector:target (E:T) cell ratio of 5:1. Group 1 clones specifically lysed both Melan-A A27L- and Melan-A_{26-35}-pulsed T2 cells, group 2 clones recognized Melan-A A27L peptide but failed to lyse Melan-A_{26-35}-pulsed T2 cells, and group 3 clones failed to significantly recognize both Melan-A peptides. At the same E:T cell ratio, lysis of empty T2 cells were used as negative control (▲). Points are means of triplicates. Data shown represent one of three experiments with similar results. In lower panel, clones were tested in TCR down-regulation experiments. T2 cells were pulsed with increasing concentrations of the natural (Melan-A_{26-35}, ■), the analogue (Melan-A A27L, ●) or an irrelevant (MAGE-3, ▲) peptide and added to effector cells to give a final E:T ratio of 1:5. After 1.5-3 h at 37°C, TCR internalization was measured by flow cytometry using an anti-TCR antibody. Figure shows one representative case for each of the three groups. Data shown represent one of two experiments with similar results.

Our data were in perfect agreement with those reported by Dutoit *et al*. on naive A2/Melan-A-reactive populations from normal donors [39] where the authors detected the same three functionally distinct groups of tetramer$^+$ clones and possibly reflect the fact that the tetramer$^+$ cells of our patient PSA contain a significant proportion of naïve cells. These results also suggested that *in vitro* Ag stimulation is the method of choice to select high avidity, self-specific cells from patients' PBMC.

Clinical perspectives: Genetic transfer of tumor-specific T cell receptors from vitiligo to melanoma

Autoimmune conditions stem from a break of tolerance to defined autoantigens and this allows for the production of high avidity antigen-specific responses. If these antigens are also relevant to tumor immunity, autoimmune cells can be exploited for tumor intervention. As the antigen specificity of T lymphocytes is dictated solely by the T cell receptor (TCR) α and β chains, genetic transfer of TCR chains may be an appealing strategy with which to impose a desirable tumor-antigen specificity onto recipient T cell populations, thus providing a promising tool for immunogene therapy of tumors [40-44]. This strategy is limited though, as it is often difficult to obtain high affinity, tumor-antigen-specific TCRs from most cancer patients. Ideally, model systems where strong and efficient responses against tumor antigens are achieved would represent a better source of therapeutic cells.

We have identified one such model in the melanoma-vitiligo dichotomy. From our results, the increased anti-tumor activity of vitiligo CTLs appeared to be mainly due to their superior TCR affinity and indeed, a characteristic shared by several different models of autoimmunity is the change in the repertoire that occurs during the course of disease progression. A way by which Ag availability can change the repertoire is through alterations in the avidity of the T cells that recognize autoantigens. A number of studies have found that, during the course of an immune response to foreign Ag, selection occurs in favor of T cells that have high affinity for Ag and that these high affinity selected cells are the ones which enter the memory pool [45-48]. It is possible that this also occurs in an autoimmune response like vitiligo. Features of affinity maturation are increasingly restricted TCR repertoires, and average increased avidity [45, 49]. In this respect, the restricted range of high avidities displayed by group 1 T cell clones, the preferential use of specific AV and BV segments and the selection for conserved CDR3 motifs in their β chain [20] suggest that affinity maturation occurred in the Melan-A/MART-1-specific T cell population during vitiligo development.

In this context, it might be interesting to design a TCR transfer approach where high affinity TCRs from vitiligo donors will be used to redirect the antigen specificity of peripheral lymphocytes from HLA-matched melanoma patients. We will address several issues which are crucial to the successful use of TCR genes for immunogene therapy, such as preservation of fine specificity, preservation of functional avidity and formation of chimeric receptors upon TCR transfer. Ultimately, this approach will be relevant to learning if protective immune functions can be enhanced for tumor immunity without excessive self-destruction.

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