Molecular and Functional Bases of Self-Antigen Recognition in Long-Term Persistent Melanocyte-Specific CD8+ T Cells in One Vitiligo Patient

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Vitiligo patients possess high frequencies of circulating CD8+ T lymphocytes specific for the melanocyte differentiation antigen Melan-A/MART-1. These self-specific T cells exhibit intact functional properties and their T cell receptors are selected for a narrow range of high affinities of antigen recognition, suggesting their important role in the pathogenesis of vitiligo. In order to understand the molecular base for this unexected, optimal T cell receptor recognition of a self-antigen, a tetramer-guided ex vivo analysis of the T cell receptor repertoire specific for the Melan-A antigen in a patient affected by vitiligo is reported. All T cell receptors sequenced corresponded to different clonotypes, excluding extensive clonal expansions and revealing a large repertoire of circulating Melan-A-specific T lymphocytes. A certain degree of T cell receptor structural conservation was noticed, however, as a single AV segment contributed to the κ chain rearrangement in 100% of clones and a conserved amino acid sequence was found in the β chain complementarity determining region 3 of various high affinity cells. We suggest that the conserved κ chain confers self-antigen recognition, necessary for intrathymic selection and peripheral homeostasis, to many synonymous T cell receptors, whereas the β chain fine tunes the T cell receptor affinity of the specific cells. In addition, we demonstrate that many high avidity T cell clones from this patient were capable of specifically lysing normal, HLA-matched melanocytes. These autoreactive clones persisted for more than 3 y in the patient’s peripheral blood. These data, together with the skin-homing potential of the clones, directly point to the in vivo pathogenic role of melanocyte-specific cytotoxic T lymphocytes in vitiligo. Key words: cytotoxic T lymphocytes/human/Melan-A/MART-1/T cell receptor/vitiligo. J Invest Dermatol 121:308–314, 2003

Vitiligo is a skin disease affecting approximately 1% of the general population and is characterized by the presence of depigmented macules and patches as a consequence of local melanocyte loss. Despite the fact that its etiology is not fully understood, vitiligo is believed to be due to an autoimmune-mediated destruction of epidermal melanocytes. Although melanocyte-specific autoantibodies have been described in vitiligo patients, their pathogenic role remains uncertain (Naughton et al., 1983; Betterle et al., 1984; Song et al., 1994; Cui and Bystryn, 1995; Kemp et al., 1997; Merimsky et al., 1999; Okamoto et al., 1998). The patchy distribution of cutaneous depigmentation and the most frequent symmetrical distribution of lesions support the hypothesis that autoimmune melanocyte damage is induced by clones of lymphocytes with affinities for specific areas of skin (al Badri et al., 1993). Evidence for the involvement of cellular immunity in the pathogenesis of vitiligo is further provided by cases of inflammatory vitiligo (Le Poole et al., 1996) and by the frequent observation of skin-homing melanocyte-specific cytotoxic T lymphocytes (CTL) in the peripheral blood of vitiligo patients (Ogg et al., 1998; Lang et al., 2001; Palermo et al., 2001).

The presence in the periphery of high numbers of CTL specific for self-antigens is not completely understood. The ability to discriminate self from nonself is a crucial property of the immune system. Self-peptides play a pivotal role in the shaping of the T cell receptor (TCR) repertoire. Selection of the TCR repertoire during thymic development begins with positive selection of T cells expressing receptors with low affinity for self major histocompatibility complex (MHC) and negative selection of the high affinity cells (Kreuwel and Sherman, 2001). The persistence in the peripheral repertoire requires homeostatic division, and this too relies on repeated TCR–MHC low affinity interactions and recognition of self-epitopes (Kreuwel and Sherman, 2001). As a consequence, only antigen-specific T cells characterized by a low avidity phenotype for self-antigens are expected to remain

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unaffected by intrathymic and/or extrathymic deletion (Liu et al., 1995; Morgan et al., 1998; Lee et al., 1999; Wang et al., 1999).

In this study, we focused on the analysis of the TCR repertoire that is specific for the HLA-A2-restricted immunodominant epitope derived from the melanocyte differentiation antigen Melan-A/MART-1 (Melan-A hereafter). Melan-A is a 118 amino acid membrane protein of unknown function, expressed by normal melanocytes, by most fresh melanoma samples, and by about 60% of melanoma cell lines (Valmori et al., 1998; Skipper et al., 1999). The immunodominance of Melan-A antigen, in association with HLA-A2, has received particular attention from the immunotherapeutic standpoint, as this HLA allele is predominant in the North American Caucasian population, and Melan-A reactivity is frequent in tumor-infiltrating lymphocytes from HLA-A2 melanoma patients. A specific allele-ligand combination capable of unique interaction with the potential of the mature human TCR repertoire appears to be the major contributor to this immunodominance (Bettinotti et al., 1998). Vitiligo patients have been demonstrated to possess high frequencies of Melan-A-specific circulating CTL (Lang et al., 2001; Palermo et al., 2001). These autoreactive T cells exhibited intact functional properties associated with antimelanocyte activity, as indicated by their consistent ability to undergo in vitro expansion and to lyse HLA-matched melanoma cell lines. Their TCR were selected for a narrow range of high avidities of antigen recognition. In order to understand the molecular base for this unexpected, optimal TCR repertoire, for HLA-A2 in one patient.

MATERIALS AND METHODS

Patient

PSA is a female HLA-A*0201 homozygote patient affected by nonfamilial, nonsegmental, patchy vitiligo diffuse on the face, arms, hands, axillae, and legs. The disease affects 60% of the patient's body surface and it has been stable for more than 10 y. The patient does not suffer from any other related autoimmune disease and remained free of therapy during the follow-up period relative to this study (3 y). All investigations were performed after approval by the local institutional review board and according to the Declaration of Helsinki principles.

Cell lines

For the generation of peptide-specific T cell lines, peripheral blood mononuclear cells (PBMC) were monocyte depleted by exclusion of plastic adherent cells. PBMC 10^6 per well were cultured in complete RPMI medium in the presence of 10^-5 peptide-pulsed HLA-A2- TAP-deficient T2 cells. After 1-2 pg per ml of Melan-A peptide. Low dose (25 U per ml) interleukin-2 was added on day 3. Cells were stimulated at 3 wk intervals.

Fluorescence cell sorting and T cell cloning

PBMC were sorted into defined populations using a FACSVantage SE and data were analyzed by CellQuest software (Becton-Dickinson, Mountain View, CA). Sorting conditions are described by Mantovani et al. (2002). Immediate re-analysis of the sorted populations revealed more than 98% purity efficiency. For the cloning experiments, either sorted cells or tetramer-stimulated at 3 wk intervals.

Cytotoxicity assays

To determine avidity in antigen recognition, titration curves over a wide range of Melan-A A27L and Melan-A26-35 peptide concentrations (3 × 10^-7 nM to 5 × 10^-7 nM) were generated and avidity of the different clones was determined as the concentration of peptide required to obtain half maximal lysis ([nM]/50% of target cells in 4 h chromium release assay; at a fixed effector:target cell (E:T) ratio (5:1). Cytotoxicity was performed as described elsewhere (Palermo et al., 2001).

T cell clones were also tested for specific lysis against HLA-A2*0201 melanocytic cell lines (kindly provided by Meinhard Herlyn, Wistar Institute, Philadelphia, PA, and derived from newborn foreskins) in a 5 h chromium release assay. An HLA-A2- normal melanocyte cell line was used as a negative control. 10^5 target cells per well were mixed with appropriate amounts of effector cells to give final E:T ratios ranging between 3:1 and 8:1.

For the redirected lysis assay, 20 × 10^5 target cells were labeled with 370 μCi 51Cr for 2 h at 37°C. Erythrocytes were pulsed with B9.12.13 antibody, and FITC conjugated goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody. Triple stainings with tetramer-PE, anti-CD3-FITC, and goat antimouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as a second antibody.
TCRBV PCR profile was 2 min at 94°C, 35 cycles at 94°C for 30 s, 55°C for 20 s, 72°C for 1 min, followed by a 7 min extension. PCR amplification products were purified using JetStar gel extraction kit (Genomed, Bad Oeynhausen, Germany) and directly sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using internal AC-specific (5'-GTCACTGAGTTAGCTC-3') and BC-specific (5'-TGCTTCTGTAGGCGTCAAA-3') oligonucleotides according to the manufacturer’s instructions. Sequences were run on an ABI 377 DNA sequencer (Applied Biosystems). The TCR nomenclature proposed by Arden et al (1995) was adopted.

Blotting and oligotyping The PCR samples were separated by 1.5% agarose gel electrophoresis and alkali-blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech). Filters were prehybridized in BLOTTO solution (6 x sodium citrate/chloride buffer, 1% milk, 5 mM ethylenediamine tetraacetic acid, 0.1% sodium dodecyl sulfate) at 37°C for 3 h and hybridized overnight at 37°C with the following, internal oligonucleotides: PSAS34N 5'-TTACCGGGACTTTCTTAAACG-3', PSAS3B 5'-TCACGGGGGGACTACCAC-3', PSAS3N 5'-TAGGTCGGTGAGCGGG-3', PSAS4N 5'-GCAATCCGCAGTACGCTGGGC-3', PSAS5N 5'-GGCCGGGAGACTGCTTACTAC-3', 47 N 5'-CCACCA CCACGCCCCCGG-3'.

RESULTS

Functional analysis of tetramer-guided ex vivo sorted cells from one HLA-A*0201 vitiligo patient reveals three groups of Melan-A-specific clones with variable avidity of antigen recognition A blood sample was obtained after signed informed consent from the vitiligo patient PSA in October 2000. Freshly isolated PBMC were stained with fluorescent HLA-A2 tetramers complexed with the immunodominant Melan-A peptide (A2/Melan-A tetramers) and anti-CD8 monoclonal antibody directly ex vivo, without any antigen-specific stimulation, and analyzed by flow cytometry. 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 naïve (CD45RA+CD28+, 57%), effector (CD45RA-CD28+, 15%), and memory cells (CD45RA+CD28+, 24%) (data not shown). The CD8+ tetramer+ population was isolated by flow cytometry cell sorting and sorted cells were more than 98% pure (not shown). The sorted population was immediately cloned by limiting dilution, and 29 clones, all staining positive to the tetramers (not shown), were functionally characterized. Avidity of antigen recognition was assessed by titrating both Melan-A A27L analog and Melan-A26-35 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-A26-35 pulsed T2 cells; group 2 clones that recognized Melan-A A27L peptide but failed to specifically lyze Melan-A26-35 pulsed T2 cells; and group 3 clones that failed to significantly recognize the two Melan-A peptides (Table I and Fig 1a). Redirected lysis experiments were performed to ascertain functionality of the clones belonging to group 3. Only one clone (clone 14) was found to be nonfunctional (not shown). TCR downregulation upon peptide encounter was found to perfectly correlate with the functional avidity of the clones (Fig 1b for some representative cases), supporting the above classification.

Table I. Avidity of A2/Melan-A tetramer+ clones from one vitiligo patient

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<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tr>
<td>PSA clones</td>
<td>A27L</td>
<td>Melan-A_{26-35}</td>
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<tr>
<td>31</td>
<td>0.3</td>
<td>4.7</td>
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<td>33</td>
<td>1.2</td>
<td>213</td>
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<td>21</td>
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<td>20</td>
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*Non-functional in redirected lysis experiments.

Molecular characterization of the peripheral TCR repertoire specific for the Melan-A antigen To study the TCR repertoire displayed by the CD8+ A2/Melan-A tetramer+ T cells, cDNA obtained from the 29 clones was subjected to RT-PCR using a panel of 32 AV-specific and 24 BV-specific oligonucleotides together with one AC-specific and one BC-specific primer. Surprisingly, all clones analyzed (28 of 29; for one clone the TCR-β rearrangement could not be determined) expressed the same rearranged AV segment (Fig 2a and Mantovani et al, 2002). In contrast, Melan-A-specific CD8+ T cells from this patient were found to rearrange many distinct BV gene segments, though the preferential use of some V elements was observed: five BV segments (BV2, BV7, BV9, BV13, and BV14) accounted for 69% of clones analyzed (Fig 2a). We then performed complementarity determining region 3 (CDR3) analysis through direct sequencing of the amplified products using an internal BC-specific primer. Recurrent clonotypes were never observed, indicating a largely polyclonal repertoire of circulating Melan-A-specific cells in this patient (Fig 2b). Clones belonging to group 1 exhibited a certain degree of TCR-β structural conservation as all but one clone (clone 43) used BJ segments belonging to cluster 2.

Previously, T cell clones had been generated from this patient (February 1999) by limiting dilution from a Melan-A-sensitized cell line (Palermo et al, 2001). The relative avidity of all clones analyzed turned out to be extremely high and to directly correlate with their in vitro antimalanocytic activity (Palermo et al, 2001). The molecular TCR composition of those clones was here defined and compared to the data obtained with the ex vivo sorted cells. All clones rearranged the same AV segment (AV2) as the sorted cells, and we found that the in vitro culture had selected...
three clonotypes (Fig 3a). One clonotype appeared to be overrepresented (five of eight clones, represented by clone 4.7 hereafter). It showed the highest reactivity against HLA-matched melanoma cell lines (Palermo et al., 2001) and, analogously to the clones belonging to group 1, it possessed a high avidity of antigen recognition for both Melan-A A27L and Melan-A26^35 peptides (Fig 3b) and used a cluster 2 BJ segment (Fig 3a). TCR down-regulation upon peptide encounter was again found to perfectly correlate with the functional avidity of the clone (Fig 3c). Interestingly, this clone contained an amino acid sequence (PGLA) in its CDR3β that was either integrally or partially shared by three ex vivo sorted cell clones belonging to group 1 (Fig 3d). This suggests that this amino acid motif confers a high TCR affinity for the Melan-A antigen.

Melan-A-specific clones from vitiligo possess skin homing capability and antimelanocyte reactivity Four of the highest avidity clones belonging to group 1 as well as clone 4.7 were assayed for their expression of CLA, which confers homing to the skin, and for their ability to lyze normal melanocytes, typically expressing low levels of the Melan-A antigen. All five clones were found to be CLA positive, though with different degrees of surface expression (Fig 4a). Importantly, these clones were also found capable of specifically lysing normal HLA-matched melanocytes (Fig 4b): a significant lysis, above 20%, was reached by four of the five clones at relatively low E:T ratios.

Long-term peripheral persistence of high avidity Melan-A-specific clones In order to evaluate the longevity in peripheral blood of the Melan-A-specific clones belonging to group 1, RNA was extracted from the patient’s PBMC collected over a 3 y period. Persistence of the individual clones was assessed through BV-specific RT-PCR followed by oligotyping with clone-specific CDR3β probes. The sensitivity of this approach, inherently very high, is limited by the relative number of PBMC used for the experiment. The calculated lower detection limit in our case is 1:15,000, meaning that, in order to be
detected, each clone must represent at least 1 cell in 15,000 CD8+ cells. As shown in Fig 5, persistence of the different clones was highly variable, ranging from less than 4 mo for clones 33 and 38 (these clones were isolated by tetramer sorting on October 2000 but they were not detectable among PBMC collected on February 2001) up to more than 3 y for clones 31, 34, and 43. Clone 47 displayed an intermediate, though not precisely limiting dilution immediately after sorting and 29 independent clones were analyzed for their AV and BV usage by RT-PCR. The percentages of clones rearranging every specific V segment are shown. The AV rearrangement of one clone could not be determined. (b) CD3εβ-junctional amino acid sequences of the 29 T cell clones are shown. One clone (#14) could not be assigned to any of the groups as it turned out to be nonfunctional (see Results). These sequence data are available from GenBank/EMBL/DDBJ under accession nos. AY900865, AY190909, and AY190915.

**DISCUSSION**

In this study, we demonstrate the presence of melanocyte-specific CD8+ T cells in the peripheral blood of an HLA-A2+ vitiligo patient and describe their molecular and functional features. The long-term persistence of some high avidity autoreactive CTL clones is also demonstrated. Those autoreactive CTL exhibit in-vitro growth characteristics and prior to this report no such studies have been performed in vitiligo, with the only exception a partial characterization limited to the A chain (Mantovani et al., 2002). Our results concerning Melan-A-specific CD8+ T cells from vitiligo reveal a large repertoire of circulating Melan-A-specific T lymphocytes, a feature shared by all the Melan-A responses previously studied by the use of tetramers in melanoma, yet a certain degree of TCR structural conservation. Although there is always a potential bias that structural conservation. Although there is always a potential bias that

Few studies have attempted to characterize the TCR usage of T cells identified by tetramers in melanoma (Valmori et al., 2000; Dietrich et al., 2001; Schrama et al., 2001; Mandruzzato et al., 2002), and prior to this report no such studies have been performed in vitiligo, with the only exception a partial characterization limited to the A chain (Mantovani et al., 2002). Our results concerning Melan-A-specific CD8+ T cells from vitiligo reveal a large repertoire of circulating Melan-A-specific T lymphocytes, a feature shared by all the Melan-A responses previously studied by the use of tetramers in melanoma, yet a certain degree of TCR structural conservation. Although there is always a potential bias that subpopulations of autoreactive T cells may display differential growth characteristics in vivo leading to the expansion of selected T cell clones, the polyclonality of the population analyzed represents a good indication for absence of such a bias in our sample. The dominant usage of the same AV segment in this patient, which

**Figure 2.** Molecular characterization of Melan-A-specific T cell clones *ex vivo* isolated from the PBMC of an HLA-A2+ vitiligo patient. (a) Sorted A2/Melan-A tetramer+ CD8+ T cells were cloned by limiting dilution immediately after sorting and 29 independent clones were analyzed for their AV and BV usage by RT-PCR. The percentages of clones rearranging every specific V segment are shown. The AV rearrangement of one clone could not be determined. (b) CD3εβ-junctional amino acid sequences of the 29 T cell clones are shown. One clone (#14) could not be assigned to any of the groups as it turned out to be nonfunctional (see Results). These sequence data are available from GenBank/EMBL/DDBJ under accession nos. AY900865, AY227209, and AY229993. (b) Avidity of clone 4.7 as measured in a standard 4 h chromium release assay against T2 cells pulsed with increasing concentrations of either the natural Melan-A26-35 peptide (2), or the analog Melan-A A27L peptide (4), at an E:T ratio of 5:1. This cell clone was found to lyze with high avidity both Melan-A A27L pulsed T2 cells (avidity 0.3) and Melan-A26-35 pulsed T2 cells (avidity 12.7). At the same E:T ratio, lysis of empty T2 cells was used as negative control (A). Points are means of triplicates. Shown is one of three experiments with similar results. (c) Clone 4.7 was tested in TCR downregulation experiments. T2 cells were pulsed with increasing concentrations of the natural (Melan-A26-35, 1) or the analog Melan-A A27L peptide (2), at an E:T ratio of 5:1. After 1.5 h at 37°C, TCR internalization was measured by flow cytometry using an anti-TCR monoclonal antibody. Shown is one of two experiments with similar results. (d) CD3εβ-junctional amino acid sequences of the Melan-A-specific clones sharing a conserved amino acid consensus motif.

**Figure 3.** Molecular characterization of Melan-A-specific T cell clones previously isolated from a Melan-A-sensitized T cell line from the same HLA-A2+ vitiligo patient. PBMC had been isolated from the vitiligo patient PSA in February 1999 and T cell clones were obtained by limiting dilution from a T cell line obtained from these PBMC upon three rounds of peptide-specific stimulations. (a) AV and BV usage was determined for eight clones by RT-PCR. CD3εβ-junctional amino acid sequences are shown. These sequence data are available from GenBank/EMBL/DDBJ under accession nos. AY900865, AY227209, and AY229993. (b) Avidity of clone 4.7 as measured in a standard 4 h chromium release assay against T2 cells pulsed with increasing concentrations of either the natural Melan-A26-35 peptide or the analog Melan-A A27L peptide, at an E:T ratio of 5:1. After 1.5 h at 37°C, TCR internalization was measured by flow cytometry using an anti-TCR monoclonal antibody. Shown is one of two experiments with similar results. (d) CD3εβ-junctional amino acid sequences of the Melan-A-specific clones sharing a conserved amino acid consensus motif.
we know to be a conserved feature of all Melan-A-specific populations we have studied so far (Mantovani et al., 2002), is particularly striking and might reflect a structural constraint imposed on the TCR for engagement with Melan-A peptides presented by HLA-A2. This, presumably low-affinity, interaction mediated by the $\alpha$ chain would be necessary for intrathymic positive selection and peripheral homeostatic proliferation of all Melan-A-reactive naïve T cells. On the other hand, the presence of a conserved amino acid motif in the CDR3$\beta$ of some high avidity cells, together with the selective use of certain BJ and, to a lesser extent, BV segments, might suggest an important role of the $\beta$ chain in fine-tuning TCR affinity of the specific cells. From this standpoint it is intriguing that a high avidity Melan-A-specific clone, present in this patient at the first evaluation, had 20 mo later been substituted by other clones with comparable avidity and sharing many TCR-$\beta$ similarities.

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 (Palermo et al., 2001), 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 analog pulsed cells. Surprisingly, a significant proportion of ex vivo sorted tetramer$^+$ T cell clones failed to recognize the two Melan-A peptides. Possibly, these clones exhibited some degree of specificity for Melan-A antigen, sufficient to bind A2/Melan-A tetramers, but their functional avidity was too low to be detected in a functional assay. Although we cannot formally demonstrate it, we believe that these clones represent essentially naïve cells that are specific for some of the many Melan-A epitope mimics described so far (Loftus et al., 1996; Rubio-Godoy et al., 2002) and that they do not participate in the antimelanocyte immune response in this patient. This would explain the presence of a conspicuous fraction of naïve A2/Melan-A tetramer$^+$ cells in a patient with a long-lasting disease. Our data are in perfect agreement with those recently reported by Dutoit et al. (2002) on naïve A2/Melan-A-reactive populations from normal donors, 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 suggest that in vitro antigen stimulation is the method of choice to select high avidity, self-specific cells from patients’ PBMC.

The patient we examined in this work had a clinically stable disease during the entire follow-up period. Accordingly, the
fraction of tetramer\(^+\) cells that had signs of antigen encounter in vivo were predominantly of the memory phenotype, with only few bona fide effectors. Different Melan-A–specific CD8\(^+\) clones showing variable longevity were apparently detectable. This could suggest that the repertoire of autoimmune T cells continues to evolve even when the disease is apparently stable, presumably due to the persistence of sufficient amounts of the sensitizing antigen. Alternatively, we might have failed to detect all clones at each time point due to sensitivity limitations of the technique we used. In this case, the autoimmune repertoire would be more stable and maintained by a fixed pool of long-lived memory cells.

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 antigen 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 antigen, selection occurs in favor of T cells that have high affinity for antigen and that these high affinity selected cells are the ones that enter the memory pool (Busch and Pamer, 1999; Rees et al., 1999; Savage et al., 1999; Amrani et al., 2000). It is possible that this also occurs in an autoimmune response. Features of affinity maturation are increasingly restricted TCR repertoires and average increased avidity (McHeyzer-Williams and Davis, 1995; Busch and Pamer, 1999). 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 might suggest that affinity maturation occurred in the Melan-A/MART-1–specific T cell population during vitiligo development.

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