Abstract

Recent studies in mouse models have suggested that genetic transfer of tumor antigen-specific high affinity T cell receptors (TCR) into host lymphocytes could be a viable strategy for the rapid induction of tumor-specific immunity. A previously proposed approach for the isolation of such TCRs consists in circumventing tolerance to self-restricting HLA/peptide complexes by deriving them from PMBCs of allogenic donors. Towards this aim, we used fluorescent HLA-A2 class-I/peptide soluble multimers to isolate A2-restricted CD8+ T cells specific for a previously described Melan-A peptide enhanced analog (Melan-A 26-35 A27L) from an HLA-A*0201 (A2) negative donor. We isolated two distinct groups of Melan-A 26-35 A27L-specific clones. Clones from the first group recognized the analog peptide with high avidity but showed very low recognition of Melan-A parental peptides. In contrast, clones from the second group efficiently recognized Melan-A parental peptides. Surprisingly however, most clones recognized not only A2+ Melan-A+ targets, but also A2+ Melan-A- targets suggesting that they can also recognize endogenous peptides other than Melan-A. In addition, one clone showed full cross-recognition of an antigenically unrelated peptide. Together, our data show that HLA-A2/peptide multimers can be successfully used for the isolation of allorestricted CD8+ T cells reactive with tumor antigen-derived peptides. However, as the cross-reactivity of these apparently peptide-specific allorestricted TCRs is presently unpredictable, a careful in vitro analysis of their reactivity to the host's normal cells is recommended.

Introduction

An overwhelming body of experimental evidence documenting both humoral and cellular responses to an increasing number of tumor-associated antigens has clearly demonstrated immune recognition of cancerous cells by the autologous human host. It has been shown that tumor cells can be destroyed by T lymphocytes that specifically recognize, through clonotypically unique TCRs, short protein fragments derived from tumor-
associated proteins (mostly non-mutated self-proteins) which are displayed on the surface of tumor cells bound to major histocompatibility complex (MHC) molecules. This knowledge has led to an increasing number of ongoing clinical trials of cancer vaccination worldwide utilizing tumor antigen-derived immunogens in various formulations.

To date, however, the most successful form of tumor-specific immunotherapy in humans has been the infusion of allogenic donor lymphocytes for the treatment of otherwise fatal hematological malignancies. This treatment has been effective in inducing complete responses in patients with chronic myeloid leukemia who had relapsed after allogenic stem cell transplantation (1, 2). Recently, this approach has also been used to treat patients with metastatic solid tumors (3). Despite their efficacy, immunotherapy approaches based on the use of allogenic lymphocytes are not widely applicable because of a potentially devastating complication known as graft-versus-host disease (GVHD) mediated by alloreactive donor T cells. However, while tumor regression after allogenic transplantation often correlates with the development of GVHD, graft-versus-tumor reaction (GVT) can also occur in the absence of GVHD suggesting that the two responses may not necessarily be mediated by the same effector cells (3, 4). This has suggested the possibility of dissociating the beneficial GVT reaction from the detrimental GVHD reaction by raising allorestricted immune responses against peptide determinants that are preferentially or exclusively expressed on tumor cells. As tolerance to self-antigens is MHC-restricted (5, 6), allorestricted tumor antigen-specific clones should exhibit increased functional avidity and tumor reactivity when compared to their self-restricted counterparts (7, 8).

Examples of allorestricted tumor-specific CTLs have previously been reported both in mice and humans (9, 10, 11). The possibility of infusing these CTLs for cancer therapy in vivo has also been explored in a murine model (10). CTLs specific for an mdm-2-derived peptide, overexpressed in RMA lymphoma cells of the H-2b haplotype and reactive against tumor but not normal cells were isolated from H-2d mice. Interestingly, mdm-2-specific allorestricted CTL clones significantly delayed tumor growth in vivo when co-injected with lymphoma cells into H2b recipients, but tumor protection was limited temporally as allogenic CTLs were eventually rejected. This indicates that, even though some cancer patients that are conditioned with irradiation and immunosuppressive drugs before bone marrow transplantation could concomitantly accept MHC-mismatched CTLs, adoptive transfer of allogenic tumor antigen-specific CTLs into immunocompetent hosts is unlikely to represent a valid approach to obtain a sustained control of cancer growth. Recent studies in mouse models, however, suggest that genetic transfer of selected TCR genes into the host's autologous lymphocytes could constitute a viable strategy to implement the use of allorestricted tumor antigen-specific high affinity TCRs for cancer immunotherapy (12).

A major obstacle to the simple isolation of tumor antigen-specific CTLs, including those of allogenic origin, has been their generally low frequency, combined with the absence of methods allowing their direct isolation. Due to the development of fluorescent HLA-class-I/peptide soluble multimeric complexes (multimers) (13, 14), it is now possible to directly visualize and isolate peptide-specific CD8+ T lymphocytes present at low frequency within heterogeneous lymphocyte populations. This method has been used extensively during the last few years to isolate self-restricted CD8+ T cells specific for a variety of antigens, including numerous tumor antigens. A recent report has documented the first attempt to isolate allorestricted T cells using HLA/peptide multimer selection (15).

The results of the present study further demonstrate that different categories of allorestricted T cells exist and can be isolated by multimer-guided cell sorting, thus underlining the need for an extensive functional characterization of the clonal populations of interest. In this study we report the functional characterization of HLA-A*0201 (A2) restricted CD8+ T cells specific for the previously described Melan-A 26-35 peptide analog A27L (16) isolated from an A2- donor using A2/peptide multimers incorporating A27L (A2/Melan-A multimers). Some of the isolated clones were specific for peptide A27L and very poorly cross-recognized Melan-A parental peptides, whereas other clones recognized Melan-A parental peptides with a functional avidity similar to that previously reported for Melan-A-specific tumor-reactive CTL clones. However, we found a general lack of correlation between the ability of the CTLs to recognize Melan-A peptides and tumor reactivity. Surprisingly, most clones were able to recognize not only A2+ Melan-A+ but also A2+ Melan-A- tumor targets. In addition, one of these clones was able to efficiently cross-recognize an unrelated A2-restricted peptide derived from the influenza
Results

The Melan-A gene (17), expressed by normal melanocytes and by the majority of fresh melanoma samples, codes for an immunodominant peptide, Melan-A 26(27)-35, frequently recognized by tumor-reactive A2-restricted CTLs. Using A2/peptide multimers incorporating the Melan-A 26-35 enhanced analog A27L (16) we have previously analyzed the frequency and phenotype of A2/Melan-A multimer+ CD8+ T cells among PBMCs of normal donors and melanoma patients (18). We reported that, in contrast with the low frequency of most single epitope-reactive T cells in the preimmune repertoire (below multimer detection limits), an exceptionally high proportion (up to 1 in 1000, mean frequency among 10 healthy donors, 0.07%) of naive CD8+ T cells specifically bind A2/Melan-A multimers in A2+ donors. In addition, we detected a proportion of A2/Melan-A multimer+ CD8+ T cells in A2- donors that was lower than that found in the A2+ group, but significantly higher than that found using A2/peptide multimers containing an influenza matrix (Flu-Ma 58-66)-derived peptide (18). For this study, we selected an A2- donor (DR) in which a population of A2/Melan-A multimer+ CD8+ T cells was clearly detectable ex vivo at a relatively high frequency (0.05% of CD8+ T cells, Figure 1A). Similarly to what was previously found in A2+ donors, these cells exhibited a naive phenotype (CD45RA+ CCR7+, not shown). PBMCs from DR were stimulated in vitro with A27L-pulsed T2 cells. After 1 week, Melan-A multimer+ CD8+ T cells were present in the culture at a higher proportion (5-fold) than ex vivo (Figure 1B). This in vitro selected Melan-A multimer+ CD8+ T cell population was isolated by cell sorting and further expanded in vitro either as a bulk culture (Bulk DR, Figure 1C) or under limiting dilution conditions as previously reported (19). After an additional two weeks of in vitro culture, the sorted bulk population was exclusively composed of A2/Melan-A multimer+ CD8+ T cells (Figure 1C). In addition, from the limiting dilution cultures, we obtained eight A2/Melan-A multimer+ CD8+ clones.

The ability of the Melan-A multimer+ CD8+ T cell population from DR to recognize Melan-A peptides and tumor targets was assessed in CTL assays (Figures 2 and 3). The Bulk DR population was able to recognize peptide A27L with a relatively high avidity (50% maximal recognition at 10 pM). In addition, it recognized Melan-A parental nona- and decapeptides (Melan-A 27-35 and 26-35, respectively) with an efficiency comparable to that previously found for a self-restricted tumor-reactive CTL clone derived from a melanoma patient (clone LAU 203/17, Figure 2). Mass spectrometric analysis of naturally processed Melan-A peptides (20), together with functional data of tumor cell recognition (21), indicates that the nonapeptide Melan-A 27-35 is the main peptide species present on melanoma cells. The Bulk DR efficiently lysed A2+ Melan-A+ tumor targets (Me 275), in agreement with its ability to efficiently recognize peptide Melan-A 27-35 and similarly to clone LAU 203/17. However, at variance with the latter which failed to significantly lyse A2+ Melan-A- tumor targets (NA8) in the absence of exogenously added Melan-A peptides, Bulk DR lysed A2+ Melan-A- tumor targets similarly to A2+ Melan-A+ tumor targets (Figure 2).
**Figure 1. Detection and isolation of circulating A2/Melan-A multimer+ CD8+ T cells from PBMCs of healthy donor DR.** (A) PBMCs from DR were stained with the indicated A2/peptide multimersPE and anti-CD8 mAbFITC. (B) PBMCs were stimulated in vitro with the Melan-A 26-35 peptide analog A27L (1 µM), cultured for one week and stained as in A. A2/Melan-A multimer+ CD8+ T cells present in this culture were isolated by cell sorting and further expanded in PHA. (C) After an additional two weeks, the resulting polyclonal Melan-A monospecific allorestricted population was stained as in A. Numbers in quadrants represent the percentage of multimer+ cells among CD8+ T cells.

**Figure 2. Functional characterization of polyclonal Melan-A monospecific allorestricted CTLs.** (A) Recognition of Melan-A peptides by the polyclonal Melan-A monospecific allorestricted CTL line Bulk DR was assessed in a 51Cr release assay on T2 target cells in the presence of increasing concentrations of Melan-A parental or analog peptides. A Melan-A specific self-restricted CTL clone (LAU 203/17) was used as an internal control. (B) Tumor recognition was similarly assessed on NA8 (HLA-A2+ Melan-A-) or Me 275 (HLA-A2+ Melan-A+) tumor target cells at the indicated effector to target cell (E:T) ratio.
Figure 3. Melan-A peptides and tumor recognition of A2/Melan-A multimer+ CD8+ allorestricted CTL clones. Recognition of Melan-A peptides and tumor cell lines by A2/Melan-A multimer+ CD8+ allorestricted CTL clones was assessed in a $^{51}$Cr release assay as described in Figure 2. In addition to NA8 (HLA-A2+ Melan-A-) and Me 275 (HLA-A2+ Melan-A+), the melanoma line Me 260 (HLA-A2- Melan-A+) was also included.

We then assessed recognition of Melan-A peptides and tumor targets by the eight isolated clonal populations. The results obtained for three representative clones are illustrated in Figure 3, whereas the results obtained for all eight clones are summarized in Table 1. Some of the isolated clones (i.e. clone DR/A2B4 and DR/A1E2, Figure 3) seemed to be specific for A27L and cross-recognized Melan-A parental peptides very poorly, whereas other clones recognized Melan-A parental peptides with a functional avidity similar or superior to that previously reported for Melan-A-specific tumor-reactive CTL clones (i.e. clone DR/A2B5). However, when we assessed tumor lysis, we found a more complex situation. As was the case for Bulk DR, clones that efficiently recognized Melan-A parental peptides were not only able to recognize A2+ Melan-A+ tumor targets but also A2+ Melan-A- tumor targets. Even more surprisingly, clones that recognized A27L and not Melan-A parental peptides exhibited two distinct patterns of tumor recognition. Some failed to significantly lyse both A2+ Melan-A- and A2+ Melan-A+ tumor targets (i.e. clone DR/A2B4). However, other clones of this type efficiently lysed both A2+ Melan-A- and A2+ Melan-A+ tumor targets in the absence of exogenously added peptide (i.e. clone DR/A2B4). However, other clones of this type efficiently lysed both A2+ Melan-A- and A2+ Melan-A+ tumor targets (i.e. clone DR/A1E2). It is of note that all populations analyzed (Figure 3 and Table 1) failed to significantly lyse A2- Melan-A+ tumor targets (Me 260). In addition, they failed to significantly recognize DR self-targets. As illustrated in Figure 4, no significant killing of Epstein-Barr virus (EBV)-transformed donor cells by clone DR/A1B4 was observed, either in the absence or in the presence of peptide A27L. In contrast, DR EBV cells were specifically lysed by a CTL line specific for an influenza nucleoprotein-derived peptide (Flu-NP 44-52) recognized in an HLA-A1-restricted fashion. Similar results were obtained for both Bulk DR and other allorestricted Melan-A/multimer+ CD8+ clonal T cell populations (not shown).
Table 1. Recognition of Melan-A peptides and tumor cell lines by DR monospecific populations.

<table>
<thead>
<tr>
<th>Peptide recognition</th>
<th>Tumor recognition</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk DR</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>DR/A1B4</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>DR/A1B9</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>DR/A1D3</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>DR/A1E1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>DR/A1E2</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>DR/A2B4</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>DR/A2B5</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>DR/A2D6</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>LAU 203/17</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

a: peptide concentration (nM) required for 50% maximal lysis.
b: specific lysis of NA8 (A2+ Melan-A+), Me 275 (A2+ Melan-A+), Me 260 (A2- Melan-A+) tumor cell lines at an E:T ratio of 50:1.
c: mean fluorescence intensity with A2/peptide multimers incorporating the Melan-A26-35 A27L or Tyrosinase268-378 peptides.

Figure 4. A2/Melan-A multimer+ CD8+ allorestricted CTL clones derived from donor DR fail to significantly recognize DR self-targets. (A) The lytic activity of A2/Melan-A multimer+ CD8+ allorestricted CTL clones from DR was assessed on NA8 cells, as well as on DR-derived Epstein-Barr virus (EBV)-transformed cells as a source of autologous target cells. Data are shown for clone DR/A1B4; similar data (lack of reactivity toward DR EBV cells both in the absence or in the presence of peptide Melan-A 26-35 A27L) were obtained in the case of other DR allorestricted clones, as well as for the T cell line Bulk DR. (B) The ability of DR EBV cells to be specifically lysed by an HLA-A1-restricted polyclonal line specific for peptide Flu NP 44-52 in the presence, but not in the absence, of the latter was also assessed as an internal control.

Together, these data suggest that some of the A2/Melan-A multimer+ CD8+ allorestricted CTLs are able to recognize endogenous peptides other than Melan-A. Lack of recognition of A2- targets, including DR autologous APCs, clearly indicates that recognition of these peptides is A2-restricted. It has previously been shown that Melan-A-related sequences are abundant among proteins, including those of self-origin (22). It is therefore possible that some Melan-A-related sequences that are normally not significantly recognized by self-restricted Melan-A-specific clones could be efficiently recognized by some A2/Melan-A multimer+ CD8+ allorestricted...
CTLs, either because of high functional avidity or of a different fine specificity. However, as clones such as DR/A1E2, that failed to significantly recognize Melan-A parental peptides, efficiently recognized A2+ Melan-A-tumor targets, recognition of Melan-A unrelated sequences by some A2/Melan-A multimer+ CD8+ allorestricted CTLs is also possible.

To gain insight into the molecular basis of antigen recognition by A2/Melan-A multimer+ CD8+ allorestricted CTLs, we analyzed the recognition of peptide Melan-A 26-35 single alanine (A)-substituted variants by clone DR/A1B4 (Figure 5A) and compared the results to those obtained for the self-restricted Melan-A specific clone LAU 203/17 (Figure 2 and 5B). Recognition of single A-substituted peptide variants in Figure 5B is shown normalized for A2 peptide binding as previously assessed (23). In the case of clone LAU 203/17, substitution of most amino acids in the peptide sequence (positions 5 to 9) with A resulted in a significant decrease in recognition (100-fold or more). It is of note that similar results were previously obtained for several other self-restricted Melan-A-specific clones (23). In the case of clone DR/A1B4 the substitution of several amino acids with A also affected recognition. However, in this case, only one of the variants was poorly recognized (A4, >100-fold decreased recognition) whereas a more limited reduction in recognition (10- to 50-fold) was found for three additional single A-substituted peptide variants (A5 to A7). Of note are the remaining three variants (A8 to A10) which were recognized more efficiently than the parental peptide (10-fold or more). Together, these results are in line with previous data in the literature suggesting that recognition of antigen in the context of an allopresenting molecule is more flexible than with the self-restricting molecule (24). This analysis, however, does not allow us to determine if the altered recognition of the peptide variants is the result of the direct interaction of the substituted amino acids with the TCR or if the latter recognizes a conformation of the MHC molecule that is differentially induced by different peptide ligands. In other words, this approach does not distinguish between antigen-dependent and antigen-specific recognition.

Figure 5. A higher degree of flexibility is observed in allorestricted as compared to self-restricted antigen recognition. (A) Relative recognition of peptide Melan-A 26-35 or of single A-substituted variants was assessed in a 51Cr release assay on T2 target cells in the presence of increasing concentrations of the indicated peptides. (B) The nanomolar concentration of peptide giving 50% maximal activity was determined from the titration curve of each peptide. Recognition of single amino acid substituted variants was calculated relative to that of the parental Melan-A 26-35 peptide. For each variant, the resulting value was then divided by the corresponding relative HLA-A2 binding value determined previously in a functional competition assay.
To further investigate the specificity of antigen recognition of A2/Melan-A multimer+ CD8+ allorestricted CTLs, we analyzed the ability of the latter to recognize a panel of A2-restricted peptides derived from other tumor antigens or from viral proteins. No significant cross-recognition was found for most of the clones (not shown). However, one clone (DR/A1B9, Figure 6A) cross-recognized peptide Flu-Ma 58-66 presented by T2 cells whereas it failed to cross-recognize other A2 binding peptides (i.e. peptide Tyrosinase 368-376, Figure 6A). Peptide Flu-Ma 58-66 was recognized with a high avidity (50% maximal lysis at 1 nM) comparable to peptide Melan-A 27L, albeit the amino acid sequences of the two peptides do not have much in common (Melan-A 27L: ELAGIGILTV, Flu-Ma 58-66: GILGFVFTL). In agreement with the recognition data, A2/peptides multimers incorporating peptide Flu-Ma 58-66 specifically stained clone DR/A1B9, but not clone DR/A1B4 which served as an internal control (Figure 6B).

**Figure 6.** Clone DR/A1B9 cross-recognizes an influenza matrix peptide. (A) Recognition of peptides Flu-Ma 58-66 and tyrosinase 368-376 by clones DR/A1B9 and DR/A1B4 was assessed in a 51Cr release assay with T2 target cells in the presence of increasing peptide concentrations. Peptide Melan-A 26-35 A27L was included as an internal control. (B) Staining of clones DR/A1B9 and DR/A1B4 with multimersPE incorporating peptide Melan-A 26-35 A27L, Flu-Ma 58-66 or tyrosinase 368-376 was performed during 1 hr at room temperature, followed by incubation with anti-CD8FITC mAb for an additional 20 min at 4°C.

**Discussion**

We report here the first attempt to isolate human tumor antigen-specific allorestricted T cells by using HLA/peptide multimers containing a tumor antigen-derived peptide presented by the restricting HLA molecule. A2/Melan-A multimer+ CD8+ allorestricted CTLs were readily isolated from PBMCs of an A2- donor cultured in vitro during one week in the presence of A2+ TAP-deficient T2 cells pulsed with the peptide analog Melan-A 26-
35 A27L. When antigen recognition was analyzed in a CTL assay using T2 cells as targets, all isolated CTLs were able to efficiently recognize the peptide analog used for the in vitro stimulation. In addition, some of the CTLs significantly cross-recognized Melan-A parental peptides. When analyzing their tumoricidal activity, however, we realized that the ability of these cells to specifically lyse tumor targets did not necessarily correlate with recognition of Melan-A parental peptides. Together, the data obtained clearly indicate that a considerable fraction of A2/Melan-A multimer+ allorestricted CTLs were able to cross-recognize peptides other than Melan-A in an A2-restricted fashion. Thus, at least part of these CTLs which appeared at first sight as peptide-specific were instead functionally closer to the subset of peptide-dependent allorestricted CTLs previously described in the literature (15, 25, 26).

The molecular bases of alloreactivity have been extensively investigated. During T cell maturation, the T cell repertoire is shaped by the interaction of the TCR with self-peptide/MHC complexes on thymic antigen-presenting cells. This process results in the deletion of autoreactive T cells that recognize self-peptide/MHC complexes with a high avidity and the positive selection of those that recognize them with low avidity. In contrast, allorestricted T cells react to allogenic MHC molecules to which they have not been exposed during this selection process. The frequency of T cells able to react to alloantigens is remarkably high, ranging from 1 to 10% of peripheral T cells (27). The molecular nature of the ligands recognized by allorestricted CTLs has been at least partially elucidated. Peptide-independent, peptide-dependent and peptide-specific allorecognition have clearly been documented (15, 25, 26, 28, 29, 30, 31). Based on the reported data, several models have been proposed that mostly differ in terms of the involvement of the MHC-bound peptide in allorecognition, ranging from recognition of the peptide only and no contacts of the TCR with the MHC molecule to recognition of the MHC molecule alone and no contacts at all with the peptide (27, 32, 33).

Available crystal structures of TCR complexes with MHC/peptide ligands have substantially contributed to advance our understanding of T cell antigen recognition including allorecognition. The crystal structure of a human TCR specific for a nonapeptide derived from the human T-cell lymphotropic virus HTLV-1 presented by the A2 molecule was resolved in 1996 (34). It was shown that this TCR contacts a total of 23 residues, 7 involving the presented peptide, whereas the majority (16) directly contacts the MHC molecule. These data support the view that the interaction between the MHC and TCR is based on a large contact surface of these two components of the trimolecular complex. The third component, namely the antigenic peptide, will only modulate the pre-existing affinity of the interaction to different extents that mostly depend on the MHC contribution. If the latter is very high, the threshold for T cell activation could easily be achieved by a high number of peptides, resulting in a highly degenerate recognition. Alternatively, if the contribution of the MHC is low, only a few peptides will be able to increase the overall affinity beyond the threshold required for activation, and recognition by the TCR will appear less flexible. Thus, the affinity of each TCR for the presenting MHC molecule would determine its degree of degeneracy (i.e. the capacity to recognize a larger or smaller number of antigenic ligands). As this value is presently not measurable, the degeneracy of T cell recognition is not predictable and needs to be assessed experimentally for each T cell clone. In allorestricted T cell antigen recognition, TCRs have an altered affinity for the allogenic, as compared to the self-restricting, MHC molecule. For some TCRs this affinity is decreased whereas it is increased for others, to different extents, often resulting in a larger number of ligands that achieve the recognition threshold required for T cell activation (peptide-dependent but not peptide-specific recognition) as compared to self-restricted T cell antigen recognition. In extreme cases, the affinity of the TCR for the MHC allomolecule is increased sufficiently so as to result in activation irrespectively of the peptide bound (peptide-independent recognition).

As mentioned previously, the majority of A2/Melan-A multimer+ CD8+ allorestricted T cell clones isolated in this study appeared to be peptide-dependent, whereas some of them appeared to be thoroughly specific for the peptide analog (A27L) used for both in vitro stimulation and multimer-guided cell sorting. It is of note that we failed to isolate peptide-independent clones that should in principle bind A2/peptide multimers irrespectively of the peptide used. This is in agreement with the ex vivo frequency of A2/peptide multimer+ T cells for multimers incorporating A2-binding peptides other than Melan-A (i.e. Flu-Ma 58-66, Figure 1) in DR's CD8+ T cells being below the detection limit. Thus, either the frequency of peptide-independent allorestricted T cells is lower than
that of T cells belonging to the peptide-specific and peptide-dependent groups, or peptide-independent allorestricted T cells do not efficiently bind HLA/peptide multimers. We also failed to isolate the subset of A2/Melan-A multimer+ CD8+ allorestricted T cells that would be the most relevant for immunotherapeutic purposes, namely the ones able to recognize the naturally processed Melan-A peptide expressed on melanoma tumor cells with increased avidity but non-reactive towards A2+ Melan-A cells. Whereas this failure could simply be explained by a relatively low frequency of these cells, it remains to be demonstrated that increased avidity of recognition of Melan-A parental peptides could still result in tumor-specific recognition without leading to recognition of Melan-A related sequences expressed on normal cells and normally ignored by self-restricted CTLs. The identification of A2-binding peptides recognized by Melan-A-specific A2-allorestricted CTLs on Melan-A cells would be of great interest to further our understanding of tumor antigen allorestricted recognition.

Together, our findings clearly indicate that, because of the inherent nature of antigen allorestricted recognition, cross-reactivity of allorestricted CTLs or derived TCRs is at present unpredictable and therefore the absence of reactivity to the host's normal cells needs to be verified in vitro before immune intervention. Even under these circumstances, a limited risk of provoking some tissue-specific GVHD as the result of recognition of some tissue-specific antigens could not be excluded a priori. This small risk could nevertheless be acceptable when treating patients with advanced disease. Thus, the HLA/peptide multimer-guided approach for the isolation of tumor antigen-specific allorestricted CTLs retains some important immunotherapeutic potential. It is of note that the frequency of A2/Melan-A multimer+ T cells detectable ex vivo in A2- donors is significantly higher than that of T cells stained by A2/peptide multimers incorporating other A2-restricted tumor antigen-derived peptides (35). Thus, the isolation of allorestricted CTLs specific for tumor antigens other than Melan-A could be more laborious than expected. This work is currently ongoing in our laboratory.

Abbreviations

A2, HLA-A2

Acknowledgements

We would like to thank Dr. D. Rimoldi for providing the melanoma lines Me 275 and Me 260, Dr. P. Batard for assistance in cell sorting experiments and N. Montandon for excellent technical assistance.

References


---

**Materials and methods**

**Cells**

The HLA-A2+ human mutant cell line CEMx721.T2 (T2) (36), used both as APC for *in vitro* stimulation and as a target in 51Cr-release assays, was cultured in RPMI 1640 medium containing 10% FCS. Melanoma cell lines Me 275 and Me 260 were established at the Ludwig Institute for Cancer Research, Lausanne, from melanoma patients LAU 50 and LAU 149 respectively. The melanoma cell line NA8 was kindly provided by Dr. F. Jotereau (INSERM, Nantes, France). A2/Melan-A multimer + CD8+ allorestricted polyclonal culture (Bulk DR) and clones were isolated by A2/Melan-A multimer guided cell sorting from PBMCs from an HLA-A2- healthy donor (DR, HLA-A1/A31, HLA-B35/B51) which had previously been cultured for 7 days in CTL medium containing hrIL-2 (100 UI/ml, Glaxo, Geneva, Switzerland, kindly provided by Dr M. Nabholz, ISREC, Epalinges, Switzerland) and hrIL-7
(10 ng/ml, R&D Systems, Europe, Oxon, U.K.) in the presence of irradiated T2 cells pulsed with Melan-A 26-35 A27L peptide (1 µM). The cells were cloned by limiting dilution culture in the presence of irradiated allogenic PBMCs, PHA, and hrIL-2 as described previously (19). Clones were subsequently expanded by periodical (3-4 wk) restimulation in microtiter plates, together with irradiated feeder cells and in the presence of PHA and hrIL-2.

**A2/peptide multimers and flow cytometry immunofluorescence analysis**

PE-conjugated multimeric A2/peptide complexes were synthesized as described (13, 14). Samples were stained with multimers at the indicated dose in PBS containing 0.2% BSA during 1 hr at room temperature, washed once in the same buffer, stained with mAbs where indicated during 20 min at 4°C, washed again and analyzed by flow cytometry. Anti-CD8 mAbs were purchased from Becton Dickinson (San Jose, CA, USA). Data analysis was performed using Cell Quest™ software.

**Antigen recognition assay**

Antigen recognition was assessed in chromium-release assays. Target cells were labeled with $^{51}$Cr for 1 hr at 37°C and washed three times. For effector to target cell titration experiments, labeled target cells (1,000 cells) were incubated with effector cells at the effector to target cell ratio indicated, in the absence or presence of peptide (1 µM). Similarly, in peptide titration experiments, target cells were incubated with effector cells in the presence of the indicated peptide concentration at an effector to target cell ratio of 5:1. In all cases, chromium release was measured in the supernatant harvested following a 4 hr incubation at 37°C. The percent specific lysis was calculated as:

$$100 \times \frac{(\text{experimental} - \text{spontaneous release})}{(\text{total} - \text{spontaneous release})}$$

**Contact**

**Address correspondence to:**

Danila Valmori  
Division of Clinical Onco-Immunology  
Hôpital Orthopédique  
Avenue Pierre-Decker 4  
CH-1005 Lausanne  
Switzerland  
Tel.: + 41 21 314-01-78  
Fax: + 41 21 314-74-77  
E-mail: danila.valmori@inst.hospvd.ch

Copyright © 2002 by Danila Valmori