Dissection of T-cell Antigen Specificity in Human Melanoma

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Abstract

Tumor-infiltrating lymphocytes (TIL) isolated from melanoma patients and expanded in vitro by interleukin (IL)-2 treatment can elicit therapeutic response after adoptive transfer, but the antigen specificities of the T cells transferred have not been determined. By compiling all known melanoma-associated antigens and applying a novel technology for high-throughput analysis of T-cell responses, we dissected the composition of melanoma-restricted T-cell responses in 63 TIL cultures. T-cell reactivity screens against 175 melanoma-associated epitopes detected 90 responses against 18 different epitopes predominantly from differentiation and cancer-testis antigens. Notably, the majority of these responses were of low frequency and tumor-specific T-cell frequencies decreased during rapid expansion. A further notable observation was a large variation in the T-cell specificities detected in cultures established from different fragments of resected melanoma lesions. In summary, our findings provide an initial definition of T-cell populations contributing to tumor recognition in TILs although the specificity of many tumor-reactive TILs remains undefined. Cancer Res; 72(7); 1642–50. ©2012 AACR.

Introduction

In patients with malignant melanoma, in vitro reactivated and expanded tumor-infiltrating lymphocytes (TIL) are able to induce cancer regression upon adoptive transfer to a lymphodepleted patient in combination with high-dose interleukin (IL)-2 treatment. Currently, objective response rates of around 50% and durable complete responses up to 22% have been achieved in single institution phase II trials by adoptive cell therapy of TILs in heavily pretreated melanoma patients (1–5). The antigen-specificity has been determined for a few T-cell clones persistent after adoptive transfer of TIL cultures (6, 7), but, in general, very little is known about the antigen recognition patterns of TILs. Tumor cells express antigens that are recognized by the clonally distributed T-cell receptor (TCR) on host T cells. These antigens arise from genetic and epigenetic alterations in the tumor, resulting in an altered protein profile of cancer cells compared with the normal counterpart (8). This alteration is mirrored by the peptide-HLA (pHLA) complexes on the cell surface and may lead to the recognition and killing of cancer cells by host T cells. By taking advantage of the impressive clinical responses of adoptively transferred TILs, a dissection of T-cell specificities in these cellular products could assist in the definition of clinically relevant tumor rejection antigens. Currently, numerous clinical strategies are being developed to manipulate the antitumor reactivity of adoptively transferred T cells, for example, by TCR gene transfer or MHC-multimer enrichment (9–11). As discussed previously (12, 13), successful implementation of these therapeutic strategies will rely to a large extent on the choice of antigen. A description of tumor reactivities in TILs could assist this choice and thereby serve as a platform to transfer the success of adoptive cell therapy to other immunotherapeutic strategies.

To identify the antigen recognition pattern of TILs, we compiled a list of all described tumor-associated antigens (TAA) and made a selection for melanoma association. To screen for T-cell reactivity against this large panel of peptides, using limited amounts of TILs, we took advantage of several novel techniques that together form the basis for a high-throughput platform to identify peptide-MHC specific T-cell populations (14).

Materials and Methods

Cells

TILs were isolated from resected tumor lesions obtained from patients with malignant melanoma. Half of the lesion was used to establish uncultured TILs and the other half to generate “standard” or “young” TIL cultures. Uncultured TILs were established by leaving mechanically disaggregated tumor fragments overnight in RPMI 1640 (RPMI; Gibco) with 100 U/mL penicillin and 100 μg/mL streptomycin (Pen Strep; Gibco) at 37° C and 5% CO2, and the following day, cell suspensions of TILs were cryopreserved at −150°C in human serum (Sigma) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Standard and young TIL cultures were generated with an
expansion protocol adapted from refs. 1, 15, and 16. For details, see Supplementary Materials and Methods.

Blood samples from melanoma patients were collected at the time of surgery, and peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on lymphoprep (Axis-Shield PoC) and cryopreserved at −150 °C in fetal calf serum (FCS; Gibco) containing 10% DMSO. Tissue typing was conducted by PCR and flow cytometry. All the procedures were approved by the Scientific Ethics Committee for the Capital Region of Denmark. Written informed consent was obtained, according to the Declaration of Helsinki.

**Generation of pHLA multimers**

All peptides were purchased from Pepscan (Pepscan Presto BV) and dissolved to 10 mmol/L in DMSO. The UV-sensitive conditional ligands were synthesized as described (17–19). Recombinant HLA-A1, HLA-A2, HLA-A3, HLA-A11 and HLA-B7 heavy chains and human β2 microglobulin light chain were produced in Escherichia coli. The heavy and light chains were refolded with the conditional ligand and purified as described (20). Specific pHLA complexes were generated with the MHC peptide exchange technology as described (20). pHLA multimers were generated by multimerization with 8 fluorescent-streptavidin (SA) conjugates [SA-PE, SA-APC, SA-PE-Cy7, SA-Quantum dot (QD) 585, SA-QD605, SA-QD625, SA-QD655, and SA-QD705; Invitrogen] to obtain 27 unique color codes useful for identification of pHLA-positive T cells (21). See Supplementary Materials and Methods for details.

**T-cell staining**

All T-cell stainings were done on cryopreserved material. HLA-A2+ TILs were rested for 1 hour before incubation with 50 nmol/L dasatinib (LC Laboratories; ref. 22; see Supplementary Fig. S1) for 30 minutes at 37 °C and 5% CO2. PBMCs and HLA-A2+ TILs were not treated with dasatinib. Up to 10⁶ cells per sample were stained with 1 pHLA multimer panel (containing 27 specificities) for 15 minutes at 37 °C, following incubation with antibody mix consisting of CD8-AlexaFluor700 (Caltag) and dump channel antibodies [CD4-, CD14-, CD16-, CD19-, CD25, and CD8-FITC (Becton Dickinson, BD)] and a dead cell marker (NIR-VD, LIVE/DEAD Fixable Near-IR; Invitrogen) for 30 minutes on ice. Before analysis, cells were washed twice with PBS containing 2% FCS. Data acquisition was carried out on an LSR-II flow cytometer (BD), and data analysis was conducted with FacsDiva software (BD). The detection limit of the technique is 0.002% of CD8+ T cells according to the work of S.R. Hadrup and colleagues (21).

**Intracellular cytokine staining**

TILs were either sorted and expanded (see Supplementary Materials and Methods for details), pHLA multimer depleted (see Supplementary Materials and Methods for details), or thawed and rested overnight. Target cells [T2 cells (CRL-1992), T2-B7 cells (ref. 23), or autologous PBMCs] were pulsed separately with selected peptides for 1 hour at 37 °C and 5% CO2, and then washed twice. TILs were stimulated with autologous tumor cells or peptide-pulsed target cells at 37 °C and 5% CO2 in RPMI with 10% human serum and 6,000 U/mL IL-2 (Proleukin, Novartis). Unstimulated TILs or HIV-pulsed target cells were included as negative controls. TIL to tumor/target cell ratio was 10:1 (for each peptide specificity). After 1 hour, 1 μL/mL GolgiPlug (BD) was added and incubation continued for additional 4 hours. Cells were harvested, stained with anti-CD3 and anti-CD8 (BD), fixed and permeabilized, and stained with anti–IFN-γ, anti–TNF-α, and anti–IL-2 (eBioscience and BD). See Supplementary Materials and Methods for details on target cells and fixation and permeabilization of the cells.

**Results**

**Generating a "universal" list of melanoma-associated T-cell epitopes**

With the purpose of screening TILs for recognition of all described melanoma-associated CD8+ T-cell epitopes, we generated a list of all TAAs published to date (version 2.0, updated until January 1, 2011). We used a combination of currently available databases: the Cancer Immunity database (24), the CTpedia database (25), and a previously published antigen list (8), combined with an intensive literature search. We included only T-cell epitopes with a defined minimal epitope sequence and HLA restriction, and evidence that the peptide is processed and presented on tumor cells. We compiled a database of 230 TAAs, grouped according to their expression profile/origin into 4 different classes of antigens: (i) differentiation antigens: antigens with an expression pattern that is restricted to defined differentiated cell types; (ii) cancer-testis/oncofetal antigens: antigens expressed specifically in testis, fetal, or germ line cells, but reexpressed in cancer; (iii) mutation antigens: antigens arising from specific mutations or translocations; and (iv) overexpressed antigens: antigens expressed in various normal tissues, but specifically overexpressed in cancer. Within the different groups of antigens, several epitopes are derived from alternative open reading frames (ORF) or alternative splicing events. Although these peptides may differ in immunologic properties, due to a limited thymic expression, they were classified according to the expression pattern of the canonical ORFs. Distribution of the 230 TAAs according to these criteria resulted in 17 differentiation antigens, 50 cancer-testis/oncofetal antigens, 39 mutation antigens, 116 overexpressed antigens, and 8 antigens of unknown origin/characteristics (Fig. 1A). Within these 230 antigens, a total number of 573 different HLA class I restricted T-cell epitopes were identified. The epitopes are distributed between 12 different HLA-A alleles, 21 HLA-B alleles, and 8 HLA-C alleles, but with a very significant overrepresentation of HLA-A2 and HLA-A24 (Fig. 1B). The complete list of antigens and T-cell epitopes is provided in Supplementary Table S1.

For this study, we aimed to select all antigens of potential relevance in melanoma, including both (i) antigens described to be immunogenic in melanoma patients, and (ii) antigens described in relation to other malignancies, but for which expression of the antigen has also been observed in melanoma. To define the second group of antigens, we used public available data on protein expression. An antigen was positively selected for melanoma association if any of the 3 given criteria...
that the level of evidence for the clinical relevance of the number of yet undescribed T-cell epitopes. We specified that there is probably a large number of yet undescribed T-cell epitopes. We specifically note that the level of evidence for the clinical relevance of the epitopes does vary strongly.

**Figure 1.** Classification of TAAs and HLA distribution of T-cell epitopes. A, the 230 TAAs grouped in 4 classes. B, HLA distribution of the 573 T-cell epitopes. C, the 120 melanoma-associated antigens ranked according to their potential relevance in melanoma: level 1 (88 antigens), immunogenicity of the antigen has been described in melanoma; level 2 (32 antigens), the expression profile revealed a strong expression in melanoma; and level 3 (20 antigens), the expression profile revealed a weaker expression in melanoma.

**Dissecting T-cell reactivity in TILs with pHLA multimers**

To screen TIL cultures for reactivity against a compiled list of melanoma-associated epitopes, we used combinatorial encoding of pHLA multimers (21). Collections of pHLA complexes were generated by UV-induced peptide exchange (17, 18, 20). Upon project initiation, this technology platform was available for HLA-A1, HLA-A2, HLA-A3, HLA-A11, and HLA-B7, and we therefore selected the 175 melanoma-associated epitopes restricted to those HLA alleles (according to version 1.0 of the peptide database, as available by the time of synthesis). HLA-A1: 10 epitopes, HLA-A2: 146 epitopes, HLA-A3: 11 epitopes, HLA-A11: 3 epitopes, and HLA-B7: 5 epitopes (Supplementary Table S3).

We analyzed 63 different TIL cultures from 19 patients for reactivity against the peptide library of 175 melanoma-associated epitopes according to patient HLA type. TIL cultures were generated with either the “standard TIL” or the “young TIL” expansion protocol, and 23 cultures were additionally subjected to rapid expansion (REP; See Materials and Methods; refs. 1, 15, 16, 28). We included 18 virus-derived T-cell epitopes in the screen to explore the prevalence of virus-specific T-cell reactivity in TIL samples (Supplementary Table S4). All peptides were divided into sets of 27, MHC multimers were generated, and these were labeled with 27 unique color codes and analyzed in 1 sample. An example of a full flow cytometric data set for 1 set of 27 HLA-A2-restricted pHLA multimers is given in Supplementary Fig. S2A. We detected T-cell responses against 18 different melanoma-associated T-cell epitopes and 9 virus-derived T-cell epitopes (Table 1). An example of each melanoma-associated T-cell epitope response detected is shown in Supplementary Fig. S2B. Altogether, we detected 90 T-cell responses against melanoma-associated antigens in TIL cultures from 15 of the 19 patients (Table 1 and Supplementary Table S5). Cultures in which we did not detect responses were all from HLA-A*02 patients and therefore involved HLA alleles for which only few epitopes have been described. The melanoma-associated responses were dominated by T cells specific for antigens from the differentiation and the cancer-testis/oncofetal groups of antigens. Thus, compared with the large representation of overexpressed antigens in the peptide library, few responses were detected against T-cell epitopes from this group of antigens. Noticeably, 3 of the 4 responses found against overexpressed antigens (AIM-2,5GS, Gnt-VV5,9P.9, and PRDX5A,5A) as well as 2 of the cancer-testis antigen–restricted responses (NY-ESO-1,ML,M and NY-ESO-1,APR) were derived from antigens expressed by alternative ORFs.

Three selected T-cell populations were sorted to generate epitope-specific T-cell cultures. These were tested for peptide recognition by intracellular cytokine staining (ICS) measuring IFN-γ, TNF-α, and IL-2 secretion. Approximately half of the pHLA multimer-specific T cells secreted cytokines upon recognition of peptide-loaded T2 cells and a similar cytokine secretion was detected for autologous tumor recognition (when available; Fig. 2). Together, these data indicate that the pHLA multimer+ T cells that we observe in TILs are both functional and relevant for tumor cell killing.
Table 1. Overview of all melanoma- and virus-associated T-cell responses detected in TIL cultures

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days in culture</th>
<th>Tumor origin</th>
<th>HLA</th>
<th>Culture</th>
<th>Antigen-Specific T Cells in Melanoma TILs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt 5</td>
<td>29</td>
<td>SC</td>
<td>HLA-A2, A11</td>
<td>M1</td>
<td>MART-1 ELA, gp100 IMD, Tyrosinase SSD</td>
</tr>
<tr>
<td>Pt 8</td>
<td>24</td>
<td>LN</td>
<td>HLA-A2</td>
<td>M2</td>
<td>MAGE-A1 G60, MAGE-A1 IR, NY-E60-1 M, M</td>
</tr>
<tr>
<td>Pt 11-2a</td>
<td>24</td>
<td>SC</td>
<td>HLA-A2, A3</td>
<td>M3</td>
<td>Gp100 YLE gp100 RLP, MAGE-A1 KTW, MAGE-A1 RVR</td>
</tr>
<tr>
<td>Pt 12</td>
<td>39</td>
<td>LN</td>
<td>HLA-A1</td>
<td>M4</td>
<td>gp100 IMD, gp100 SSP, Tyrosinase SSD</td>
</tr>
<tr>
<td>Pt 13</td>
<td>25</td>
<td>LN</td>
<td>HLA-A1, A3</td>
<td>M5</td>
<td>NY-E60-1 M, M, gp100 YLE, gp100 RLP</td>
</tr>
<tr>
<td>Pt 14</td>
<td>47</td>
<td>LN</td>
<td>HLA-A2</td>
<td>M6</td>
<td>NY-E60-1 M, M, gp100 YLE, gp100 RLP</td>
</tr>
<tr>
<td>Pt 15</td>
<td>32</td>
<td>SC</td>
<td>HLA-A2</td>
<td>M7</td>
<td>gp100 YLE gp100 RLP, MAGE-A1 KTW, MAGE-A1 RVR</td>
</tr>
</tbody>
</table>

**Note:** The melanoma-associated responses are sorted by classification: differentiation, cancer-testis (CT), overexpressed (OE), and mutation (M) antigen. Virus-restricted T-cell responses are included in the bottom. Blue, low-frequency responses (< 0.1% of CD8+ T cells); orange, intermediate (0.1%–1% of CD8+ T cells); and pink, high-frequency responses (≥ 1% of CD8+ T cells). Grey color represents “not relevant” due to HLA discrepancy. “Days in culture” is given as days in bulk culture + days with rapid expansion. See Supplementary Table S5 for more details on frequencies.

Abbreviations: M and F, bulk culture; REP, rapidly expanded culture; Y, young TIL; Pt, patient.

1From the same patient, but biopsies from 3 different time points.

2Ten cultures from 4 patients are not shown in the table, due to no responses observed.

3For the majority of the cultures, we included both the wildtype and modified peptides for MART-1 ELA and gp100 IMD. Presented here is only results from the modified epitopes.
Variable specificities and response frequencies

In the TIL screening, we observed a very large variation in response frequencies and in the specificities detected in the different cultures. T-cell frequencies (% of CD8⁺ T cells) varied from low (<0.1%), intermediate (0.1%–1%) to high (>1%), as depicted in Table 1. However, the majority of the responses (55 out of 90) against melanoma-associated epitopes were of low frequency, and the high-frequency responses (20 out of 90) were restricted to a few frequently recognized epitopes: MART-1 ELA, gp100 YLE, gp100 KTW, AIM-2 RSD, and MAGE-A1 RVR. We detected melanoma-associated T-cell responses restricted to both HLA-A1, HLA-A2, HLA-A3, and HLA-B7, but there was a bias toward HLA-A2–restricted responses, reflecting the larger number of T-cell epitopes identified with this HLA restriction.

When comparing different cultures from the same lesion, a large variation in both the frequencies and the specificities of T-cell responses was still detected (Table 1). The TIL cultures that we tested were generated based on passive T-cell migration from fragments of resected melanoma lesions. The strong variation in T-cell composition may therefore be indicative of a stochastic variation in the number of T cells capable of clonal expansion in each fragment, or stochastic variation in the specificity of the T cells that migrated from the tumor lesion. In either case, the strong variation in composition of parallel TIL cultures must imply that antigen-specific T-cell populations in such cultures derive from very few “founder cells.”

Virus-specific T cells in TIL cultures

Virus-specific T-cell reactivity was detected against 9 different cytomegalovirus (CMV), Epstein-Barr Virus (EBV), or influenza A (FLU)-derived epitopes in TIL cultures from 10 different patients (Table 1). TIL cultures containing virus-specific T cells derived from both subcutaneous (SC; n = 2) and lymph node (LN; n = 8) melanoma lesions, and in comparison, the 11 TIL cultures not containing virus-specific T cells derived from 5 SC and 6 LN lesions. Based on this detection, we considered the possibility that T-cell responses in TILs to a large extent reflect the T-cell reactivities present in peripheral blood. To investigate this hypothesis, we screened a number of corresponding PBMC samples for T-cell responses against all the virus-derived epitopes (Supplementary Table S4) and the melanoma-associated epitopes that were recognized in the TIL cultures. This analysis showed that when a virus-derived response was detected in TILs, this response was, in all but 1 case, also detectable in PBMCs (though with variation in T-cell frequency; Fig. 3). In contrast, the majority of the melanoma-associated responses detected in TILs were not detected in PBMCs (data not shown); however, in a few cases (patient 5 and 8) melanoma-restricted responses (MART-1 ELA and GnT-V VLP) were detected in PBMCs but not in TILs. Together, these data indicate that a specific infiltration and/or preferential expansion of tumor-specific T cells do take place at the tumor site.

Changes in TIL composition during culture

To analyze whether the composition of TILs can change during culture, we screened uncultured TILs from the same lesions as used for standard TIL generation. When comparing
the specificity and frequency of tumor-specific T-cell responses in matched samples, we observed that many responses varied in frequency, and that some T-cell responses were only detectable in the uncultured TILs (1 response) or in the cultured TILs (6 responses; Fig. 4A). It should be noted that due to limitation in cell numbers, 2 of the HLA-A2+ uncultured TILs were screened only using a selective panel of 54 melanoma-associated T-cell epitopes, including the specificities found in cultured TILs. With this caveat in mind, there seemed to be no overall trend to specific gain or loss of reactivity during culturing, but large fluctuations in T-cell response magnitude. The fact that several responses became detectable only after culture is, again, indicative for a very low number of "founder cells" for each antigen-specific T-cell population.

We further tested whether the rapid expansion process used to generate clinical quantities of TILs could induce further changes in the TIL composition. We screened 4 cultures for melanoma-associated T-cell reactivity before and after rapid expansion and observed a decrease in frequency of tumor-specific T cells, but an increase in frequency of virus-specific T cells (Fig. 4B). Thus, there seemed to be a preferential expansion of virus-specific T cells compared with tumor-specific T cells during rapid expansion. We tested if this difference in expansion capacity could be explained by the phenotype of the antigen-specific T cells, but no major differences in the expression of typical CD8+ T-cell phenotypic markers were detected (Supplementary Table S6). The majority of the TILs expressed CD45RO and CD28, they all had a low expression of CCR7 and CD62L, and CD25 expression varied between the different TILs (4.2%–79% of pHLA multimer+CD8+ T cells). CD57 had a tendency toward a higher expression on virus-specific T cells (mean: 49% of pHLA multimer+CD8+ T cells) compared with tumor-specific T cells (mean: 16% of pHLA multimer+CD8+ T cells), and was the only marker that showed differential expression between virus- and tumor-specific T cells. The differences between bulk and REP cultures were not striking, but there was a tendency toward a decreased CD28 (2 of 3) and increased CD25 (3 of 3) expression after REP for tumor-specific T cells. This was not observed for virus-specific T cells.

Limitations in the library of described melanoma-associated antigens

We screened the TIL cultures for reactivity against antigens restricted to HLA-A1, HLA-A2, HLA-A3, HLA-A11, and HLA-B7. Although the majority of patients express at least one of these alleles, this approach is still limited by the fact that few T-cell epitopes have been identified for other HLA alleles than HLA-A2. In addition, the contribution of patient-specific antigens to the tumor recognition potential of TILs has not been established. This led us to investigate whether there was any correlation between the frequency of T cells recognizing
epitopes from the melanoma T-cell epitope library and the recognition of autologous tumor. Cytokine production (IFN-γ, TNF-α, and IL-2) from 6 different TIL cultures was analyzed after stimulation with either an autologous tumor cell line or peptides pulsed on T2 cells (transfected with relevant HLA) or on autologous PBMCs. We observed for 5 out of 6 patients a strong correlation between the melanoma peptide reactivity and reactivity against autologous tumor (R² = 0.93), whereas the last patient showed high frequent tumor recognition (53.2% of CD8⁺ TILs), but no reactivity against peptides (corresponding with a response against NY-ESO-1MLM of only 0.03% of CD8⁺ TILs), and therefore no overall correlation between melanopeptide–restricted reactivity and autologous tumor recognition could be established (R² = 0.0004; Fig. 5A).

To test whether a fraction of the tumor reactivity could be explained by the TILs recognizing epitopes from our T-cell epitope library, we depleted the pHLA multimer⁺ cells from the TIL cultures and measured cytokine secretion upon stimulation with autologous tumor. We compared TIL cultures positively selected for melanoma-pHLA–specific cells, depleted for melanoma-pHLA–specific cells, nondepleted and depleted for HIV-pHLA–specific cells (Fig. 5B). We observed that melanoma-pHLA–specific T cells exhibited a better recognition of autologous tumor cells than the total TIL population in 2 out of 3 cultures in which these cells were of sufficient numbers (patients 3, M4; 7, M5; and 11-1, REPM3). However, only for 2 cultures (patient 11-1, REPM3 and 7, M5) it seems that pHLA multimer depletion of melanoma peptide–specific T cells resulted in decreased tumor recognition. We confirmed from 1 culture (patient 3, M4) that the majority (72%) of the pHLA multimer–specific TILs were depleted (data not shown).

Together, these data indicate that TILs specific for the peptides in the melanoma library do recognize tumor and therefore contribute to tumor cell recognition. However, the effect of this contribution, relative to the rest of the TIL population, varies between cultures.

Discussion

To dissect the antigen specificities of TILs, we generated a peptide library including all known TAAs and the HLA class I epitopes described within their sequence. We selected antigens of relevance for melanoma, either based on immunologic findings or melanoma-restricted expression patterns. The library of melanoma-associated antigens, as well as the full TAA library, shows a great bias to HLA-A2–restricted epitopes. HLA-A24 is also well represented, but all other HLA-A and in particular HLA-B and HLA-C alleles are very poorly represented. Thus, there is a general need for description of T-cell epitopes having other restrictions than HLA-A2 and HLA-A24.

The dissection of T-cell reactivity in in vitro expanded melanoma TILs that we present here shows that these cellular products contain a variety of low-frequency antigen-specific T-cell populations and a small number of high frequent populations. The majority of the T-cell responses detected were directed toward differentiation antigens (MART-1 and gp100). Notably, we detected only few responses against overexpressed antigens, even though these epitopes represent the largest group of the described TAAs (77 of the 175 T-cell epitopes).

It could be speculated that the relative lack of T-cell reactivity against this group of antigens is due to stronger tolerance mechanisms as a consequence of the broader expression pattern of these antigens. In this light, it is of interest that 3 out of 4 of the responses that were detected against overexpressed antigens were directed toward epitopes encoded by alternative ORFs (AIM-2RSD, GnT-VVLP-9, and PRDX5AMA), and it may be speculated that T-cell tolerance against this subgroup of epitopes is less profound.

A substantial fraction of the tumor-associated epitopes (e.g., from MART-1, gp100, p53, and cyclin B1) binds with relatively low affinity to HLA class I—presumably reflecting T-cell tolerance toward higher affinity ligands from self-proteins. To
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control for the detection of T cells specific for low-affinity epitopes, we included both the unmodified and affinity-optimized variants of MART-1 (EAA and ELA) and gp100 (ITD and IMD) in the TIL screen. T-cell responses against both variants were detected at comparable levels. In line with this, we have previously shown that pHLA complexes generated by the UV-exchange technology can be used to successfully generate pHLA multimers even for very low-affinity complexes (17).

Strikingly, TIL cultures from different fragments gave rise to very different TIL compositions, indicating that few “founder T cells” were responsible for the responses detected. Furthermore, comparison of T-cell frequencies between uncultured and bulk-cultured TILs revealed large fluctuations. Interestingly, during REP the frequency of T cells specific for melanoma-associated antigens decreased and the frequency of virus-specific T cells increased. Taking into account the massive increase in cell number that occurs in this process (1,000- to 3,000-fold), it is apparent that the absolute number of tumor-specific T cells is still enhanced. Nevertheless, a reduction in frequencies of tumor-reactive T cells by REP may translate into an equal reduction in the contribution of tumour-reactive T cells after reconstitution of the lymphodepleted patient. The different expansion of virus- and tumor-specific T cells could not fully be explained by the phenotype of the cells. There was a trend toward higher CD57 expression on virus-specific T cells compared with tumor-specific T cells, and also a decreased CD28 and increased CD25 expression after REP for tumor-specific T cells. This may, in part, explain the superior proliferative capacity of the virus-specific T cells; however, the phenotype observed here might have changed, due to the long-term culture, and hide a potential difference in the original phenotype. It was previously shown by Berger and coworkers that the original phenotype of a T cell determines the ability of the T cell to persist and reacquire the phenotypic and functional properties of memory T cells in vivo in an adoptive transfer setting in macaques (29). It is likely that the virus-specific T cells were derived from less differentiated cells than the tumor-specific T cells and they may have an intrinsic capacity to expand better. It may be speculated that a massive expansion of T cells before adoptive transfer may not necessarily be required for clinical efficacy; as reported previously, very few short-term cultured virus-specific T cells were sufficient to clear a CMV infection (11) and to treat EBV-positive Hodgkin’s Disease (30).

The antigen specificity or frequency of melanoma-associated peptide-restricted T-cell responses in TIL samples showed no correlation with disease stage, previous treatment, time from primary diagnosis, or patient age (Supplementary Table S7). This is in line with data obtained in a comparable study of the antigen-specific composition of the TIL products used for clinical adoptive transfer applications to melanoma patients [Kvistborg, P. and colleagues, accepted for publication (31)].

T cells present at the tumor site were reactive to both tumor-associated and virus-derived antigens (and possibly others), but compared with T cells present in peripheral blood there was a significant overrepresentation of tumor-specific T cells at the tumor site, and virus-specific T cells were only occasionally detected. Moreover, virus-specific responses were typically (though not exclusively) found in cultures established from LN resections, in which these cells would normally be expected. This indicates a considerable local expansion and/or specific infiltration/retention of tumor-specific T cells, as is also supported by previous studies (32).

It remains an open question, to what extent our present knowledge enables us to describe the tumor reactivity observed in TIL cultures. In 5 out of 6 TIL cultures, we find a correlation between melanoma peptide reactivity and autologous tumor recognition; however, although peptide-reactive T cells do recognize autologous tumor, this barely affected the tumor recognition of pHLA-depleted TIL populations. This barely affected the tumor recognition of pHLA-depleted TIL populations. Thus, although the melanoma peptide–reactive T cells are not directly responsible for the majority of the tumor recognition, the frequency of melanoma peptide–reactive TILs may be used as a surrogate marker for total reactivity in the majority of cultures.

Taken together, it is evident that the long list of melanoma-restricted T-cell epitopes available today is insufficient to fully explain the TIL-mediated tumor recognition. Additional data on tumor-associated T-cell epitopes restricted to a broader set of HLA alleles and the contribution of patient-specific antigens would assist to fill the gap. However, despite the low frequency of melanoma peptide–specific T-cell populations, this technology platform offers a mean to map specific cell populations in T cell–based infusion products on the basis of a large (and continuously growing) peptide library, and to track these populations after adoptive transfer for potential correlates with T-cell persistence, tumor reactivity, and clinical efficacy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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