Oligoclonal T-Cell Receptor Usage of Melanocyte Differentiation Antigen-reactive T Cells in Stage IV Melanoma Patients

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Abstract

Ex vivo ELISPOT analysis of peripheral blood lymphocytes obtained from stage IV melanoma patients demonstrated reactivity against peptides derived from MART-1 and gp100. However, the number of reactive T cells was <1% that of total lymphocytes as detected by flow cytometry using tetrameric MHC/peptide complexes. Despite this low frequency, we were able to directly isolate these populations ex vivo by means of magnetic beads coated with MHC/peptide complexes and to subject these cells to T-cell receptor clonotype mapping. This analysis revealed that the MART-1/A*0201- and gp100/A*0201-reactive T-cell populations are composed of oligoclonal T cells that engage several T-cell receptor β chain families. Longitudinal studies using this approach may result in a better correlation between T-cell reactivity and the course of neoplastic disease.

Introduction

Recent technological advances (i.e., the introduction of tetrameric MHC-peptide complexes, ELISPOT analysis, and TCR clonotype mapping) revealed new insights into T-cell responses against viruses and neoplastic cells (1–3). For the latter, it became obvious that specific T-cell responses are spontaneously present and, as in the case of melanoma, are frequently directed against self-antigens (4). Furthermore, the magnitude of TCR-repertoire usage in antitumor T-cell responses was larger than previously anticipated (5). Still, it should be noted that most studies using TCR-repertoire analyses are limited by the lack of knowledge about the antigen specificity of overexpressed T-cell clonotypes (6–8). To date, only one such study attempted to characterize the TCR-receptor usage of melanoma-reactive T cells identified by tetrameric MHC-peptide complexes (9). However, the technique used for TCR-repertoire analysis did only allow to distinguish between different TCR families but not between individual T-cell clones. Here, we tested PBLs of stage IV melanoma patients for reactivity against MDAs MART-1 and gp100, isolated such cells using specific MHC-peptide complexes coupled to magnetic beads, and subsequently established their TCR BV region usage and clonotype composition by DGGE clonotype mapping.

Materials and Methods

Patients. Peripheral blood from two HLA-A*0201-positive males, ages 65 (patient 1) and 61 (patient 2), with stage IV metastatic melanoma were obtained after informed consent. Patients received either chemotherapy with dacarbazine alone (patient 1) or in combination with low-dose IFN α (patient 2).

ELISPOT Assay. The ELISPOT assay used to quantify peptide epitope-specific IFN-γ-releasing effector cells was described previously (2). Briefly, nitrocellulose-bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with an anti-IFN-γ antibody (1-D1K; Mabtech, Uppsala, Sweden), and nonspecific binding was blocked with AIM V (Life Technologies, Inc., Gaithersburg, MD). Lymphocytes were added at different cell concentrations together with the specific peptides and incubated overnight at 37°C. After two washes, the biotinylated detection antibody (7-B6-1-Biotin; Mabtech) was added. Its specific binding was visualized by using alkaline phosphatase-avidin together with the respective substrate (Life Technologies, Inc., ). The reaction was terminated on the appearance of dark purple spots, which were quantitated with the Alphalager System (Alpha Innotech, San Leandro, CA). The peptides used for ELISPOT assays were a modified gp100269–271 epitope, with a methionine at position 210 and a modified MART-126–35 epitope, with a leucine at position 27.

Construction of HLA-Peptide Tetrameric Complexes, Flow Cytometry, and T-Cell Sorting. A recognition site for enzymatic biotinylation using biotin protein ligase (BirA) in fusion with the 5′ end of the extracellular domains of HLA A*0201 (residues 1–275) was expressed in Escherichia coli BL21 (DE3). The recombinant protein was purified by size- (Sephadex G25; Pharmacia) and ion-exchange (mono-Q; Pharmacia) chromatography from inclusion bodies solubilized in 8 M urea. The HLA A*0201 was folded in vitro by dilution in the presence of antigenic peptides derived from gp100 or MART-1 and subsequently biotinylated as described previously (3). After gel filtration on a Pharmacia Sephadex G25 column, the protein was multimerized with neutralization-phycocyanin (Molecular Probes, Eugene, OR). The HLA A*0201 construct was a kind gift from Dr. Mark M. Davis (Department of Microbiology and Immunology, Stanford University, Palo Alto, CA). For flow cytometry, 5 × 10⁶ cells were centrifuged at 300 × g for 5 min, resuspended in 50 µl of PBS, and the gp100 or MART-1/A*0201-tetramers were added and incubated for 30 min at room temperature. Subsequently, cells were washed once with PBS and immediately analyzed by a FACScan with CellQuest software. Cell separation was performed as described previously (10). Briefly, 5 × 10⁶ streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway) were washed twice in 200 µl of cold PBS and 0.5 µg of peptide*A*0201 monomers were added, and the mixture was incubated for 15 min at room temperature. After two washes, these beads were mixed with PBLs at a ratio of 1:10 and subsequently incubated for 1 h, followed by a precipitation of bead-bound cells in a magnetic field. The precipitation step was repeated once.

TCR Clonotype Mapping by DGGE. DGGE clonotype mapping of the human TCR BV regions 1–24 has been described in detail (1). Specifically, RNA was isolated by using the Pan RNA kit I (Pan Biotech GmbH, Aidenbach, Germany) and transcribed cDNA was amplified by PCR with primers for the variable regions of the TCR β chains. Attachment of a GC-clamp to the 5′ end of the primer annealing to the constant region ensured that the amplified DNA molecules were suited for DGGE. DGGE analysis was done in 6% polyacrylamide gels containing a gradient of urea and formamide from 20–80%.

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3 The abbreviations used are: TCR, T-cell receptor; BV, β variable; DGGE, denaturing gradient gel electrophoresis; TIL, tumor-infiltrating lymphocyte; PBL, peripheral blood lymphocyte; MDA, melanocyte differentiation antigen; RT-PCR, reverse transcription-PCR.
Results and Discussion

Several studies have focused on the complexity of TCR usage of tumor-reactive T cells (7, 8, 11). However, the value of these studies was limited by the scanty characterization of the specificity of the analyzed T cells. Therefore, we performed a tetramer-guided ex vivo analysis of the complete TCR-repertoire of MDA-recognizing T cells present in the peripheral blood of stage IV melanoma patients.

We first analyzed the ability of PBLs of the two stage IV melanoma patients to react against MDA (i.e., modified MART-1\textsubscript{26–35} and gp100\textsubscript{209–217} epitopes) by measuring the specifically induced production of IFN-γ in the ELISPOT assay. As shown in Fig. 1, each spot represents a peptide-reactive, IFN-γ-producing T cell. The average number of spots per peptide was calculated using a CCD scanning device and a computer system. For patient 1, an average of 86 MART-1 peptide-specific spots per $3 \times 10^5$ cells could be detected (Fig. 1A). Patient 2 showed a strong response to the gp100-derived peptide with an average of 73.5 peptide-specific spots per $3 \times 10^5$ cells (Fig. 1B). This reactivity increased to 172 peptide-specific spots in the second sample obtained 5 months later (Fig. 1C).

Recently, it has been demonstrated that the ELISPOT assay underestimates the number of peptide-reacting T cells (12). This is due to the fact that only effector cells that can produce IFN-γ on stimulation can be detected; thus, subpopulations such as naive, exhausted, or anergic cells are missed. Therefore, we determined the percentage of peptide-reacting T cells among PBLs of the patients by flow cytometry using MART-1/A\textsuperscript{*}0201- or gp100/A\textsuperscript{*}0201-tetrameric complexes. For patient 1, 0.63% of all lymphocytes were stained with the MART-1/A\textsuperscript{*}0201-tetrameric complex (Fig. 2A). The amount of gp100\textsuperscript*0201-tetramer-detected T cells in the peripheral blood of patient 2 was 0.80% and 0.71%, respectively, in the two samples analyzed (Fig. 2, C and D). Due to the limited amount of blood available, we excluded the detailed characterization of tetramer-stained cells because we anticipated that large cell numbers would be needed for subsequent tetramer-guided isolation of MDA-recognizing T cells. Moreover, several excellent studies have already addressed this issue previously (9, 12–16).

Over the past few years, much effort has been spent on the clonal characterization of T cells recognizing melanoma-associated antigens, in general, and MDA, in particular. However, inconsistent and sometimes contradicting results were obtained. These were due, at least in part, to confounding factors inherent in the experimental strategy (e.g., the analysis of in vitro expanded T cells; Refs. 11 and 17), the restriction of the TCR analyses to only a fraction of the TCR BV repertoire (9) or the clonotype mapping of only overexpressed TCR BV families (7). To avoid these complicating factors, we characterized the clonal composition of MDA-recognizing T-cell populations directly, by ex vivo analyses. For this purpose, we applied tetramer-guided isolation in combination with RT-PCR/ DGGE-based TCR clonotype mapping. The latter technique allows the analysis of the complete TCR BV repertoire for the presence of clonal expanded T cells both in overexpressed and normally expressed BV families, even when only small numbers of cells are available.

The first series of experiments addressed the feasibility of using specific MHC-peptide complexes to isolate reactive T cells. To this end, we coated magnetic beads with MART-1/A\textsuperscript{*}0201-complexes and used these to isolate MART-1-reactive cells from a T-cell line that was obtained from TILs by in vitro expansion for several weeks in interleukin 2-containing medium. The frequency of MART-1-reactive cells was measured by peptide-induced production of IFN-γ in the ELISPOT assay before and after isolation. The assay was not performed immediately but 7 days after the isolation procedure to avoid the presence of contaminating MART-1\textsubscript{26–35} peptides in the negative controls. Before isolation, an average of 80 MART-1 peptide-specific spots per $10^5$ cells could be detected (data not shown). After isolation, this number increased to >250 spots per $1.3 \times 10^5$ cells (data not shown). Thus, this method of isolation was, indeed, highly efficient to enrich specific peptide/MHC-reactive T cells, because afterward such cells were expressed at two logs greater frequency.

These encouraging results prompted us to use magnetic beads coated with either MART-1/A\textsuperscript{*}0201- or gp100/A\textsuperscript{*}0201-complexes to sort out T cells recognizing these antigens from PBLs ex vivo without any measures of prior in vitro expansion. The yield of reactive cells by this approach ranged between 0.02% and 0.09%. Although these numbers corresponded well with the number of T cells detected with fluorescent peptide/A\textsuperscript{*}0201-tetrameric complexes by flow cytometry,
visual inspection of isolated cells revealed that some of them were labeled only with one bead. Consequently, we increased the specificity of the isolation procedure by additional washing steps so that only lymphocytes with several bound peptide/A*0201-coated beads were seen (Fig. 2B). However, these additional washing steps reduced the cellular yield to 0.008–0.023%. Immediately after isolation, the T cells were subjected to TCR clonotype mapping, which revealed their oligoclonal origin. T cells of patient 1 recognizing MART-1/A*0201 were composed of 11 clones that belonged to six different TCR BV families (i.e., 5, 6, 12, 14, 21, and 22; Fig. 3). For patient 2, T cells recognizing gp100/A*0201 included a higher number of clones, namely 19 for the first and 20 for the second time point analyzed; these T cell clones belonged to eight or nine TCR BV families, respectively (data not shown). Hence, the number of clonally expanded T cells detected among MDA-specific T cells in the peripheral blood of melanoma patients is substantially lower than the number of clonally expanded cells present in TILs comprising approximately 40–60 clonotypes spanning almost all TCR BV families (5). However, it is important to note that the additional washing steps in the isolation process might have removed some low-affinity clones. Nevertheless, the extent of the TCR-repertoire usage in the circulating T-cell population reactive against only one MDA suggests a rather tight selection of T-cell clonotypes for recruitment into the pool of TILs. This notion is based on the assumption that if the magnitude of the antigenic repertoire of melanoma as well as the number of T-cell clonotypes recognizing one individual peptide/HLA-complex are used to predict the amount of TCR BV usage among TILs, this hypothetical number would be considerably larger than what has been detected experimentally (5, 18).

The comparison of T-cell clonotypes present in different metastatic melanoma lesions from the same individual revealed that matching clonotypes were generally not detectable in more than one lesion (5). This observation indicated the predominance of localized T-cell expansions at least for the effector phase of immune responses to melanoma. The presence of MDA-reactive CD8+ T-cell clonotypes in the peripheral blood of stage IV melanoma patients raises the question why such cells are not part of the TIL repertoire and, hence, resulting in identical T-cell clonotypes being present in several metastatic sites. A possible reason for these discrepant observations may be that the analysis of peripheral blood provides only a momentary glimpse of ongoing immune responses that do not necessarily reflect the complexity and heterogeneity of a dynamic T-cell response. This concept prompted us to test whether the clonotypes composing the MDA-reactive T-cell population would change over time or whether this composition is durable. Remarkably, comparative RT-PCR/DGGE analysis of circulating gp100/A*0201-reacting T cells from patient 2, isolated 5 months apart, revealed that some of the clonotypes detected (e.g., within the BV families 12 and 17) resolved at positions in the gel which indicated identity (Fig. 4), a notion unequivocally proven by subsequent sequencing of the CDR3 region (data not shown). Therefore, it is justified to conclude that the T-cell reactivity against MDA in peripheral blood is maintained by a finite and persistent repertoire of clonally expanded T cells. However, these T-cell clonotypes, particularly the persisting ones, seem to have only a limited capacity to infiltrate the tumor microenvironment (19). This conclusion is in line with several clinical studies demonstrating a lack of correlation between T-cell reactivity measured in peripheral blood and the course of neoplastic disease (20).

In summary, we demonstrate the magnitude and complexity of cellular immune responses to defined MDA-derived T-cell epitopes in peripheral blood of melanoma patients. These findings emphasize the need to analyze not only the peripheral blood but also the tumor site, as well as secondary and tertiary lymphatic tissues, for the presence and activation status of tumor-reactive T cells to obtain more adequate insights into immune responses to solid tumors.

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References


Fig. 3. TCR clonotype mapping of MART-1-recognizing T cells from patient 1. Following MART-1/A*0201-guided isolation, T cells were subjected to RT-PCR/DGGE clonotype mapping using specific primers covering TCR BV regions 1–24.

Fig. 4. Comparative analyses of the clonal composition in BV families 12, 15, 17, and 18 of gp100/A*0201-recognizing T cells from patient 2 at two time points 5 months apart. The cells were isolated by using gp100/A*0201 complexes coupled to magnetic beads, and TCR clonotype mapping was performed by RT-PCR/DGGE. Identities of T-cell clonotypes in BV families 12 and 17 were confirmed by sequencing.


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