Recovery of CD8⁺ T-Cell Function During Systemic Chemotherapy in Advanced Ovarian Cancer

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Abstract

Immunologic approaches are emerging as new treatment options in several types of cancer. However, whereas the ability of patients to develop potent CD8⁺ T-cell responses is crucial for efficient antitumor responses, immunocompetence and T-cell function are not tested routinely in patients entering immunotherapy. The objective of our study was to monitor T-cell function in advanced cancer and during chemotherapy. CD8⁺ T-cell function of 21 patients with advanced ovarian cancer (stages III-IV) was assessed by cytokine flow cytometry following stimulation of 42 PBMC samples with a panel of synthetic viral peptides in vitro, consisting of pan-Caucasian epitopes. CD8⁺ T-cell responses were significantly lower in patients with high levels (>200 units/mL) of Ca125 (marker of tumor load and progression) than in those with low Ca125 levels (P = 0.0013). In longitudinal studies of nine patients, chemotherapy was associated with decreasing Ca125 levels in seven cases and also with improvement or maintenance of CD8⁺ T-cell function in seven cases. After the full course of chemotherapy, five of nine patients in remission displayed potent CD8⁺ T-cell responses, whereas four of nine patients in progression displayed low or decreasing T-cell responses, pointing toward a correlation between T-cell function and clinical response. Our results show for the first time that CD8⁺ T-cell function is not permanently suppressed in advanced cancer and successful chemotherapy is associated with improved antigen-specific T-cell reactivity. We suggest that functional assays determining T-cell immunocompetence can be valuable tools for optimizing cancer immunotherapy for improved clinical success. (Cancer Res 2005; 65(15): 7000-6)

Introduction

Tumor cells of epithelial origin express class I, but not class II, molecules and thus are potential targets for immunosurveillance by CD8⁺ T cells. Tumor-infiltrating T cells, detected in various types of tumor tissue, e.g., ovarian (1) or colorectal (2) cancer, often predict improved prognosis and overall survival rate, especially if the infiltrating T cells are CD8⁺ (2, 3) and functionally active (4). Tumor antigen-specific CD8⁺ T cells can control tumor growth in mouse models, and there is also evidence of therapeutic effect of T cells in human tumors (5, 6). However, functional abnormalities of T cells have been observed in numerous types of cancer, including head and neck, lung, esophageal, and breast cancers (7–10). The disappointing results of cancer vaccine trials designed to boost antitumor T-cell responses highlight the need to know more about general T-cell function in patients entering these trials, especially as such patients tend to have advanced disease with large tumor loads. The most common methods for assessing cellular immune status, such as measuring delayed-typed hypersensitivity reactions, lymphocyte numbers, CD3⁺ cell numbers, ratios of CD4⁺/CD8⁺ lymphocytes, or mitogen-induced lymphocyte proliferation (11), do not provide information about physiologic T-cell function. Measuring antigen-specific T-cell responses is a more appropriate way to gain information about T-cell function. However, there is no widely used approach or control antigen applicable for donors with various human leukocyte antigen (HLA) types (7, 12).

To be able to assess T-cell function in unselected cancer patients, we established a method using cytokine flow cytometry for detecting antigen-stimulated CD8⁺ T-cell responses at single-cell level from patients with common Caucasian HLA class I haplotypes. The method was also designed to overcome practical limitations in clinical settings, such as low cell numbers, the lack of repeated fresh blood samples from the same donor, or the use of cryopreserved cells.

T-cell responses were assessed in ovarian cancer patients, as the presence of tumor-infiltrating CD8⁺ T cells is associated with better prognosis in ovarian cancer (1, 13), predicting an important role for cellular antitumor immune responses. Although surgery and chemotherapy are effective in reducing tumor burden in the majority of ovarian cancer patients, only ~10% to 15% of patients with advanced disease will ultimately survive. Immunotherapy is being explored as a possible treatment modality targeting Ca125, a molecular marker of ovarian cancer, and newly defined tumor antigens (14).

Clinical trials with immunotherapy protocols are conducted in patients with evidence of recurrent disease after chemotherapy, without assessing their functional immunologic status. In a small study, we determined the T-cell function of 21 ovarian cancer patients in different stages of platinum-based chemotherapy. A strong correlation was found between clinical tumor responses and CD8⁺ T-cell function during, and upon completion of, chemotherapy. We suggest that monitoring general T-cell function during conventional treatment of advanced malignancies may allow better timing and ultimately more successful outcomes for adjuvant immunotherapy.

Materials and Methods

Donors, blood collection, and processing. Venous blood was collected from healthy volunteers and from ovarian cancer patients with advanced epithelial cancer [Fédération Internationale des Gynécologues et Obstétristes (FIGO) stage III or IV] following cytoreductive surgery, before,
during, and after first-line carboplatin/paclitaxel cytotoxic chemotherapy. Ethical approval was granted by the Bro Taf Local Ethical Committee and informed consent was obtained from all donors. Routine laboratory tests (full blood count, Ca125 level) at the time of blood collection were carried out at Velindre Hospital. Peripheral blood mononuclear cell (PBMC) fraction was separated from the blood by Histopaque (Sigma, St. Louis, MO) density gradient centrifugation and cells were used immediately or were cryopreserved in 20% FCS, 10% DMSO freezing mixture, and stored in liquid nitrogen. HLA typing was done by the Welsh Blood Service using a PCR–single-strand conformation polymorphism method.

Synthetic peptides. Eleven peptides, each 15-amino-acid long, were synthesized by Severn Biotech, Ltd. (Kidderminster, United Kingdom) and were at least 70% pure as determined by mass spectroscopy. The choice of viral peptides was based on published sequences and represented antigenic epitopes of cytomegalovirus, EBV, and influenza antigens, restricted by the most common Caucasian HLA class I types (Table 1). Although the core of the 15-mers were known 9-mer epitopes (15), the longer peptides may also contain further potential class I epitopes, as indicated in Table 1. The peptides were dissolved in DMSO at 5 mg/mL, aliquoted, and kept at −20°C until required.

CD8+ T-cell stimulation with peptides. Approximately 1 × 10^6 PBMC per well in 1 mL RPMI, containing 10% human AB serum (Sigma), were incubated in 48-well trays with the viral peptide mix at a concentration of 10 μg/mL. The following cytokines were added within 12 hours, as indicated in the figure legends: IFN-γ (PBL Biomedical Laboratories, Piscataway, NJ) at 1,000 units/mL; IL-12 (Peprotech, Rocky Hill, NJ) at 20 ng/mL; IL-6 (Peprotech) at 500 units/mL; IL-7 (Genzyme, Cambridge, MA) at 20 ng/mL; interleukin (IL)-2 (Chiron, Emeryville, CA) at 10 units/mL; tumor necrosis factor (TNF)-α (R&D, Minneapolis, MN) at 20 ng/mL; interleukin (IL)-2 (Chiron, Emeryville, CA) at 10 units/mL, and IL-7 (Enzyme, Cambridge, MA) at 5 mg/mL. A second stimulation was carried out 7 days later with antigen-presenting cells processed into 8- to 10-mer peptides to allow presentation on HLA blood dendritic cells. 15-mer peptides require a certain amount of presentation by antigen-presenting cells present in PBMC, such as monocytes, B cells, and dendritic cells. 15-mer peptides require a certain amount of processing into 8- to 10-mer peptides to allow presentation on HLA class I molecules. The cytokine environment was designed to promote peptide presentation by antigen-presenting cells present in PBMC, such as monocytes, B cells, and dendritic cells. 15-mer peptides require a certain amount of processing into 8- to 10-mer peptides to allow presentation on HLA class I molecules. The cytokine environment was designed to promote peptide presentation by antigen-presenting cells present in PBMC, such as monocytes, B cells, and dendritic cells. 15-mer peptides require a certain amount of processing into 8- to 10-mer peptides to allow presentation on HLA class I molecules. The cytokine environment was designed to promote peptide presentation by antigen-presenting cells present in PBMC, such as monocytes, B cells, and dendritic cells.

Preparation of B lymphoblastoid cell lines. BLCLs were developed according to established methods using EBV-containing supernatant from B95.8 cells in the presence of cyclosporin A. Cell lines were negative for Mycoplasma in monthly tests using a PCR method (Cambio, Ltd., Cambridge, United Kingdom).

Flow cytometry. Surface labeling of PBMC with the following conjugated antibodies was carried out on ice according to the manufacturer’s instructions: CD3-PECy5, CD4-CyChrome, CD8-CyChrome, and CD8-PeCy5 (all from BD PharMingen); CCR7-PE (R&D), CD45RA-FITC, and CD62L-PE (Serotec, Oxford, United Kingdom).

For cytokine flow cytometry, the cultures were washed in PBS, fixed, and permeabilized with Intraprep reagents (Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. The cells were then labeled with FITC-conjugated IFN-γ, CyChrome, or PE-Cy5-conjugated CD8 and PE-conjugated CD3 antibodies (all from BD PharMingen) at 37°C for 45 minutes. Cells were analyzed within 2 hours on a FACSscan (Becton Dickinson, Mountain View, CA) flow cytometer using CellQuest software. For cytokine flow cytometry, 100,000 events were collected. CD8 expression versus IFN-γ positivity was analyzed on cells double gated by size (FSc and SSC) and CD3 expression, as described (16).

Cytokine bead array. Th1/Th2 cytokines were detected with a cytokine bead array kit (BD PharMingen). Following the manufacturer’s instructions, 50 μL aliquots of undiluted tissue culture supernatant from wells of T-cell stimulation on day 7 of in vitro culture (before restimulation with BLCL) were tested for levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10 on a FACSscan flow cytometer.

Results

Optimization of CD8+ T-cell functional assay: cytokines and antigen-presenting cells. The purpose of these experiments was to establish a method capable of detecting T-cell stimulation in vitro from PBMC of cancer patients without the need for generating dendritic cells in advance. The cytokine environment was designed to promote peptide presentation by antigen-presenting cells present in PBMC, such as monocytes, B cells, and dendritic cells. 15-mer peptides require a certain amount of processing into 8- to 10-mer peptides to allow presentation on HLA class I molecules. The cytokine environment consisting of IFN-γ, IL-1β, and IL-6 was more efficient in supporting T-cell stimulation than other combinations of cytokines, without resulting in nonspecific T-cell activation (Fig. 1A, d). IFN-γ alone or in combination with IL-1β did not significantly improve T-cell stimulation (Fig. 1A, b, c). The combined cytokine treatment also enhanced T-cell stimulation from the PBMC of ovarian cancer patients by 41% (41 ± 28%, n = 4, compared with stimulation in the absence of cytokines). Interestingly, the combination of IL-2 and IL-7 was also beneficial in two of four cancer patients, but not in

### Table 1. Peptides representing common viral antigenic epitopes, restricted by the most frequent Caucasian HLA class I types

<table>
<thead>
<tr>
<th>HLA restriction elements*</th>
<th>Score†</th>
<th>Sequence‡</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1, A26, B8</td>
<td>31, 24, 20</td>
<td>IQMCTELKSLDYEGR</td>
</tr>
<tr>
<td>2</td>
<td>A1, A2, A26, B51</td>
<td>35, 20-21</td>
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<tr>
<td>3</td>
<td>A2, B51</td>
<td>30, 20</td>
<td>LTKGILGFVTLTYP</td>
</tr>
<tr>
<td>4</td>
<td>A2</td>
<td>28</td>
<td>IQNAGLCTIVAMLLE</td>
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<tr>
<td>5</td>
<td>A2, A3</td>
<td>21, 30</td>
<td>SAIIRGSAHKSL</td>
</tr>
<tr>
<td>3</td>
<td>A3, B7, B27</td>
<td>19-25</td>
<td>BKTPVTVGGGAMAGA</td>
</tr>
<tr>
<td>7</td>
<td>A2, B7, B51</td>
<td>21-22</td>
<td>SQAPLPCWDPVLPE</td>
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<td>27, 23</td>
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<td>B44</td>
<td>15</td>
<td>QEFNDANDYIFQA</td>
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<td>11</td>
<td>A1, A2, A26, B44</td>
<td>16-24</td>
<td>QTEENLDFVRMFV</td>
</tr>
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*Published and/or predicted.
†Predicts the strength of binding of peptides and the probability of being processed and presented by a given HLA allele, according to the SYFPEITHI algorithm (http://www.syfpeithi.de/Scripts/MHCServer.dll/home.htm).
‡Ref. (15) and Influenza Sequence Database, http://www.flu.lanl.gov/review/epitopes.html.
healthy donors, to improve peptide-specific in vitro T-cell responses (not shown). Thus, the combination of IFN-γ, IL-1β, and IL-6 was used routinely in all our T-cell stimulation experiments. For the restimulation step of T cells with peptides, an HLA-typed bank of BLCL (>30 lines) developed in our laboratory, was used by closely matching the HLA class I types of T cells and BLCL in all experiments. However, it may not be necessary to maintain an extensive BLCL bank, as we show here that the method can be simplified (Fig. 1B). The efficiency of a mixture of three BLCLs, representing most of the main HLA class I types listed in Table 1, or autologous PBMC, to support restimulation (Fig. 1B) was tested. The “standard” mix of BLCL was as efficient in restimulating T cells as closely matched or autologous BLCL, from a healthy donor (Fig. 1B, left), and an ovarian cancer patient (Fig. 1B, right). Autologous PBMCs were significantly inferior in providing support for T-cell restimulation in both cases, but especially in the ovarian cancer patient. These results indicate that T-cell function, using the method described here, can be monitored with the help of only a few BLC lines; however, in this paper, we used closely matched BLCL for all experiments.

**Comparative results obtained with cytokine flow cytometry and cytokine bead array for IFN-γ.** The results obtained with cytokine bead array (Fig. 2A), compared with those of cytokine flow cytometry (Fig. 2B), indicate that the amount of IFN-γ produced by in vitro–stimulated T cells was a direct reflection of the number of activated CD8+ T cells. Although the cytokine bead array kit did not provide useful data detecting other cytokines such as TNF-α, IL-2, IL-4, IL-6, and IL-10 on day 7 following in vitro peptide stimulation of PBMC, it was able to detect the same pattern of IFN-γ response as the cytokine flow cytometry method, but without the extra restimulation step. Plotting the results together (Fig. 2C) shows a positive correlation between the data obtained by the two separate methods. We used cytokine flow cytometry in all subsequent experiments as it provides extra information at a single cell level about the phenotype of IFN-γ producing cells and is more cost effective.

**Impaired CD8+ T-cell function from peripheral blood mononuclear cell of ovarian cancer patients with large tumor load (high Ca125).** The clinical relevance of monitoring T-cell function was assessed from 42 frozen PBMC samples of 21 ovarian cancer patients with FIGO stage III to IV disease, following debulking surgery, and at various stages of platinum-based chemotherapy. The patients were selected randomly from those used closely matched BLCL for all experiments.

**Figure 1.** Optimization of method. A, the presence of IFN-γ, IL-1α, and IL-6 provides the best cytokine milieu for antigen-specific CD8+ T-cell stimulation from PBMC with peptides in vitro. White columns, T-cell stimulation from the PBMC of a healthy donor in the absence of peptides but in the presence of cytokines; Black columns, T-cell stimulation in the presence of viral peptides and cytokines as follows. a, no cytokine; b, IFN-γ; c, IFN-α + IL-1β; d, IFN-α + IL-1β + IL-6; e, IL-1β; f, IL-6, g, TNF-α; h, TNF-α + IL-1β + IL-6; j, IL-2 + IL-7. Percentage of IFN-γ-producing CD8+ T cells are shown as determined by cytokine flow cytometry. Statistical analysis (t test): *P = 0.0568; **P = 0.014. The figure is representative of at least two repeated experiments. B, closely matched BLCL and a standard BLCL mix are similarly efficient in restimulating T-cell responses. In these assays, T-cell restimulation from a healthy donor (A1, A26, B8, B51; left) and an ovarian cancer patient (A3, A30, B18, X; right) was carried out using the following types of peptide-pulsed cells: closely matched or autologous BLCL (white columns); a standard mix of three BLCL representing most of the main HLA class I types listed in Table 1, or autologous PBMC (black columns). Columns, means from triplicate samples; bars, SD. The background IFN-γ production in the absence of peptides was <0.4% in all groups and is not shown. A representative of three similar experiments is shown.

**Figure 2.** Detection of T-cell activation with cytokine bead array or cytokine flow cytometry yields similar results. A, cytokine bead array results obtained with the supernatants of in vitro–stimulated T cells of a healthy donor 7 days after stimulation in the presence of IFN-γ alone without or with peptides (first and second graphs) or in the presence of IFN-γ, IL-1β, and IL-6, without or with viral peptides (third and fourth graphs, as indicated). The groups of cytokines measured, starting from the top, is as follows: IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. The numbers represent the mean fluorescence intensity (m.f.i.) of the IFN-γ plots (in black boxes). Spontaneously produced and exogenously added IL-6 levels are shown in line 3 from the top. The levels of other cytokines were not elevated. B, T cells, from the same wells that provided the supernatants for the cytokine bead array assay above, were restimulated overnight in the presence of autologous BLCL, peptides, and Golgi Plug for cytokine flow cytometry assay. The cells in the right upper quadrants are CD8+ T cells that produced IFN-γ upon peptide stimulation. C, comparative analysis of cytokine bead array versus cytokine flow cytometry data. IFN-γ production, detected by cytokine bead array, is expressed as pg/mL and plotted as symbols (black circles; right axis), whereas the background IFN-γ producing cells are shown as columns bars (left axis). Representative of three repeated experiments.
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attending chemotherapy clinics. Eleven of 21 patients provided serial (two or three) samples at different stages during their treatment. In vitro CD8+ T-cell responses were plotted against each patient’s Ca125 level at the time of donating blood samples (Fig. 3A). Whereas patients with low levels of Ca125 had a wide range of responses, similar to that observed in healthy donors (not shown), those with high Ca125 levels responded poorly to the antigen challenge. The difference between the CD8+ T-cell function of patients with low (<30 units/mL; n = 19) or high (>200 units/mL; n = 12) Ca125 levels was statistically significant (P = 0.0013; Fig. 3B). High levels of Ca125 indicate a large tumor load, but only significant serial changes in its level serve as a prognostic tool in the majority of patients (17, 18). Patients with medium levels of Ca125 (30-200 units/mL; n = 11) represented a mixed group, where single readings of Ca125 levels may have belonged to either increasing or decreasing series of Ca125 readings (not analyzed). The individual recall T-cell responses varied widely in this group and although the mean value of T-cell responses fell between those of the other two groups (Fig. 3B), the differences were not significant. No correlation was found between Ca125 levels and the number of lymphocytes, WBC, neutrophils, eosinophils, monocytes or basophils, and CD4/CD8 ratios, respectively (not shown).

CD8+ T-cell function during chemotherapy correlates with clinical responses. We wanted to determine how well the changes in CD8+ T-cell responsiveness reflect the serial changes of Ca125 levels and clinical changes in individual patients during chemo-
therapy. T-cell responses in nine patients were tested before, during, and after chemotherapy. As a control, T-cell responses from six healthy donors were also tested from frozen PBMC samples collected at three different time points. The inter- and intra-assay variations of the method were determined from the samples of healthy controls. The interassay coefficient of variation of the method from healthy donor HD1 (Fig. 4A) was 7.5%, as determined from three different samples analyzed in three separate experiments. The average intra-assay variation was 20% as calculated from the coefficients of variation of all healthy donor samples shown on Fig. 4A (18× triplicates). The longitudinal monitoring of recall peptide-specific T-cell responses in healthy donors revealed that these responses (biological variation) are fairly stable over an average time of 14 months. Statistically significant changes in the percentages of responding T cells were only observed in two cases (Fig. 4A: donor HD4, second versus first sample, and donor HD5, third versus first sample). In donor HD6, the peptides were not immunogenic as T-cell activation was not higher (2-fold increase is generally accepted as the cutoff point for positivity) than the background in any repeated samples. Similar analysis in patients who responded well to chemotherapy as confirmed by a drop in their Ca125 level or by computed tomography scan revealed that their CD8+ T-cell reactivity in vitro was either increased (OC1, OC2, and OC5; Fig. 4B) or maintained at a significant level (OC3 and OC4; Fig. 4B). The Ca125 level of patient OC5 was elevated before surgery (not shown). Her Ca125 was low when entering chemotherapy and remained low throughout, whereas the originally low T-cell response rose and remained high during chemotherapy. Elevated T-cell responses during chemotherapy confirm that tumor load rather than platinum-based chemotherapy represents a significant negative effect on memory T-cell responses. These five patients were stable at 86, 58, 33, 36, and 31 weeks, respectively, after their first chemotherapy treatment (mean time to progression has not been reached at 48.8 weeks). Three further patients (OC6, OC7, and OC8; Fig. 4C) initially also responded to chemotherapy, as judged by their decreasing Ca125 levels, but deteriorated after the third round of chemotherapy treatment, as indicated by increased Ca125 level or increased tumor mass detected by a computed tomography scan. Following a transient increase, CD8+ T-cell responses declined (OC6 and OC7; Fig. 4C) or remained low from the beginning of chemotherapy (OC8) in these patients. One patient with residual tumor (OC9) did not respond to chemotherapy and lacked T-cell responses to the viral antigen mix. In patients OC7 to OC9, the impairment of T-cell function preceded the clinical relapse. These four patients have progressing tumors (mean time to progression was 25.2 weeks).

No differential effect of chemotherapy on naïve (CD45RA⁺CCR7⁻) or memory (CD45RA⁻CCR7⁻ or CCR7⁻) T cells. The sensitivity of memory T cells to chemotherapy may be different from that of naïve T cells, potentially resulting in an increase of the relative frequency of memory T cells. We studied whether this is the case by analyzing PBMC samples of three patients before chemotherapy, of the same three patients during (after three cycles of) chemotherapy, and of three healthy donors. The proportion of CD45RA⁻CD3+ cells did not decrease during chemotherapy and was not lower than that in healthy controls (Fig. 5A). More detailed analysis using three-color immunofluorescence for the expression of CD4 or CD8 molecules and CD45RA and CCR7 markers was conducted to determine the proportion of naïve (CD45RA⁺CCR7⁻), central memory (CD45RA⁻CCR7⁻), effector memory (CD45RA⁺CCR7⁻), and terminal effector...
There was no significant decrease in the proportion of naïve CD8^+ T cells nor any significant changes in the proportion of the other subsets of T cells (Fig. 5B). No significant differences were observed in the subsets of CD4^+ T cells either (not shown).

**Increased proportion of CD62L^-cells during chemotherapy.** The proportion of CD62L^-CD3^+ cells was significantly elevated in the PBMC of patients when undergoing chemotherapy (Fig. 5C), compared with that in the same three patients before treatment (P < 0.05) or in healthy controls (P < 0.01). The presence of CD62L^-T cells indicates an ongoing T cell activation during platinum-based chemotherapy that is not reflected using CD45RA as a marker. The frequency of T regulatory cells was also studied using three-color labeling of CD4, CD25, and GITR molecules, but only very low levels were found in the PBMC in general, and there was no difference between the groups of donors (not shown).

**Figure 5.** Chemotherapy does not alter the ratio of naïve versus memory subpopulations (based on CD45RA, CCR7 staining), but increases the proportion of CD62L^-T cells. PBMC of three ovarian cancer patients before (stripes) and during chemotherapy (cross-hatch), and of three healthy donors (horizontal lines) were analyzed for surface markers. One additional Ca125 measurement 1 to 2 months after completing chemotherapy treatments, and the third after the completion of six chemotherapy treatments. One additional Ca125 measurement 1 to 2 months after completing chemotherapy treatment is also included. Blood sample was not collected at that occasion for T-cell assay. Statistically significant improvements of T-cell responses (P < 0.05, t test) were observed in samples from patients OC1, OC2, and OC5 during chemotherapy compared with T-cell responses before treatment. All five patients were progression-free at the time of the submission of the manuscript (86-31 weeks after entering chemotherapy).
Discussion

During the past 10 years, the number of cancer immunotherapy approaches has been steadily increasing together with evidence about the importance of antitumor immune responses in several types of tumor and in different stages of the disease. Constantly improving results of clinical trials suggest that immunotherapy will become part of the antitumor armory in the future. However, there is also a growing recognition of the need for generally applicable cellular immune monitoring assays that are able to measure relevant aspects of the immune response against tumors (11, 19, 20). The list of peptide epitopes from tumor antigens for T-cell recognition is continuously expanding, but the focus from HLA-A2–restricted epitopes is shifting only slowly. Moreover, even in the T-cell studies in HLA-A2 patients, a generally applicable and widely used positive control is still lacking. These limitations hamper the development of comprehensive studies about the cellular immune status of cancer patients and may also be responsible for the suboptimal design and relatively low success rate of some immunotherapy protocols. As a preliminary study to an immunotherapy clinical trial in ovarian cancer patients, we developed a method measuring CD8+ T-cell function of posttherapy patients undergoing chemotherapy. We describe the monitoring method here and provide evidence that the results obtained are clinically relevant correlating, as they do, with the clinical responses in ovarian cancer patients.

The method is based on the use of antigen-presenting cells present in PMBC, as preparing dendritic cells is not always practical from patients where the sample size is limiting. The cytokines that gave the best results when included in the in vitro T-cell stimulation cultures were IFN-α, IL-1β, and IL-6. IFN-α is a powerful inducer of blood myeloid dendritic cell differentiation from precursors (21, 22), and IFN-α–induced dendritic cells promote IFN-γ production and cytotoxic activity of natural killer cells and T cells, especially when present together in the same in vitro culture (23) probably due to “cross-talk” between the two cell types. IL-1β promotes IL-12 production and immunostimulatory functions of dendritic cells (24, 25). IL-6 is involved in macrophage differentiation, antagonizes transforming growth factor-β induced inhibition of CD3 function (26), and dendritic cell–derived IL-6 is needed for optimal T-cell function by allowing T cells to overcome the suppressive effect of CD4+CD25+ regulatory T cells (27). IL-1β and IL-6 in combination provide signaling to stimulate an IL-2–dependent pathway of T-cell proliferation (28). In our experiments, supplementing the in vitro cultures with IL-6 resulted in improved CD8+ T-cell responses and increased sensitivity to lower concentrations of peptides (not shown). The simultaneous application of the above three cytokines for the stimulation of T cells in PMBC-derived cultures has not been described before.

The control antigen consisted of 11 viral peptides, restricted by at least six common HLA class I alleles. The sequences were based on published 9-mer epitopes of cytomegalovirus, EBV, and influenza virus antigens (15). However, the published 9-mer epitopes were only used as cores and they were extended into 15-mers (both at COOH and NH2 terminals), which provided further potentially immunogenic overlapping epitopes. Thus, the estimated cumulative frequencies of class I alleles represented >100% in Caucasian individuals, and we found that 12 of 14 healthy individuals responded to this peptide mix. The individual levels of response in healthy donors varied greatly (0.5-23%); however, in most donors, the biological variation remained relatively stable as tested through several months or even a year. The 15-mer peptides need trimming before they can bind class I MHC molecules as 8- to 10-mer peptides. This probably occurs via proteasomal processing by antigen-presenting cells but also by membrane-associated peptidases (29); thus, the method may also represent a measure of antigen processing competence of cells in PMBC.

The clinical relevance of this functional assay of CD8+ T cells was tested in ovarian cancer patients undergoing chemotherapy. In epithelial ovarian cancer, Ca125, a mucin produced by many human tumors, is widely accepted as a diagnostic marker (30). Although Ca125 level is not an absolute indicator of tumor load, significant or serial decrease or increase of its level can serve as a prognostic tool in the majority of patients (17). We found a significant inhibition of CD8+ T-cell function in patients with high Ca125 levels (>200 units/mL) when testing 42 samples from 21 ovarian cancer patients with advanced disease. Nine individual patients were followed up for detecting serial changes of Ca125 levels and CD8+ T-cell responses during the course of adjuvant chemotherapy in longitudinal studies. The experiment for each individual was carried out, with samples obtained at different time points, in a single assay. As the biological variation of CD8+ T-cell responses to the standard panel of viral peptides in healthy donors was relatively low, the changes in the levels of T-cell responses in patients can be attributed to the effects of chemotherapy. The results of the longitudinal studies further confirmed our initial observation, showing that during tumor progression CD8+ T-cell responses can become seriously suppressed and successful chemotherapy is able to reverse this suppression. A limited number of studies have reported negative effects on T-cell responses in ovarian cancer patients or by ovarian tumor cells (31, 32); however, no systematic study has been carried out revealing that T-cell function is not indiscriminately low in all patients with advanced ovarian cancer but changes dynamically, influenced most of all by the tumor. Our results indicate that there are windows of opportunity during the course of the disease when patients have functionally active T cells. Being able to detect these periods may provide opportunities when adjuvant immunotherapy can be successfully applied. Determining the exact mechanism of how the immunosuppression, associated with tumor progression, is lifted during chemotherapy in ovarian cancer patients was outside the scope of this paper. However, our preliminary data indicate that although the relative proportions of naïve, central, and effector memory T cells (33) remain unchanged during chemotherapy, there is a significant increase in the proportion of CD62L−CD3+ T cells, indicating the appearance of T cells with activated phenotype (34) and effector function (35). The exact nature of these T cells and their potential role in maintaining disease-free survival is presently being analyzed.

This is the first study monitoring general CD8+ T-cell function in cancer patients during systemic chemotherapy. Importantly, the results reveal a correlation between T-cell function of patients and their clinical response, changing the view of general T-cell immunosuppression in cancer to a dynamic relationship between tumor progression and immune responsiveness. Better understanding of this relationship should enable us to design better immunotherapies for cancer.

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