Detection of CD4 T-Cell Responses to a Tumor Vaccine by Cytokine Flow Cytometry


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Abstract

Cytokine flow cytometry (CFC) is a simple and powerful method for measuring antigen-specific T-cell responses by detection of intracellular cytokine staining. We applied this method to the detection of CD4 T-cell responses to tumor vaccines. Patients with multiple myeloma were immunized against their autologous tumor immunoglobulin idiotype, using antigen-pulsed dendritic cell vaccination. Blood samples were drawn before and after vaccination, and CFC and proliferation assays were performed. For CFC, whole blood was incubated overnight with antigen in the presence of costimulatory antibodies to CD28 and CD49d. The blood was then treated with EDTA, erythrocytes were lysed, and leukocytes were fixed, permeabilized, and stained for intracellular cytokines [tumor necrosis factor-α (TNF-α) or IFN-γ], CD4, and CD69. Cells were analyzed by flow cytometry and cytokine-producing CD69+ cells enumerated as a percentage of CD4 cells. Of nine patients analyzed, three demonstrated detectable CFC responses to tumor immunoglobulin and/or keyhole limpet hemocyanin (KLH) after vaccination. One of these patients responded only to KLH, whereas the other two responded to both tumor immunoglobulin and KLH. Most responses were detected with both TNF-α and IFN-γ, but one patient’s KLH response was detected only with TNF-α. There was a positive, but not strong, correlation of cytokine responses with proliferative responses to KLH. Although further follow-up and correlation with clinical outcome is needed, CFC may represent a simple yet detailed assessment of T-cell frequencies and subsets responding to cancer vaccines.

Introduction

The detection of cellular immune responses to vaccination is vital to the assessment of vaccine efficacy, particularly in cases where cellular immunity is important for protection. This is likely to be the case for most cancers (1, 2). Traditional methods of assessing cellular immune responses include proliferation assays using [3H]thymidine incorporation and cytotoxicity assays using 51Cr release. These assays are labor intensive and generally require 5–7 days of in vitro restimulation to detect measurable responses. As a result, they do not accurately reflect the frequency of precursors present in the original blood sample, because proliferation and apoptosis have occurred to unknown degrees over time in culture. Simpler, more rapid, and more quantitative assays of antigen-specific T cells should therefore improve the quality of immunological data that can be obtained in clinical trials of cancer vaccines.

Newer assays that seek to address this need include enzyme-linked immunospot assays (3, 4), MHC-peptide tetramer staining (5), and CFC (6, 7). A comparison of relevant features of these assays is shown in Table 1. Of these assays for identifying antigen-specific T-cell frequencies, CFC offers a unique combination of advantages. These include a functional readout (as opposed to phenotypic staining), relatively fast turnaround time, compatibility with whole blood (thus minimizing sample preparation time), and perhaps most importantly, a multiparameter readout that provides additional qualitative information about the responding cells. Such information can include T-cell subsets (CD4 and CD8), expression of activation, and memory cell markers, among others.

Previous studies have used CFC to detect responses to a variety of infectious disease antigens including CMV (6–10), EBV (11), other herpes viruses (10), HIV (12–14), tetanus toxoid (15), and Brucella (16). Additionally, responses to tumor-associated melanoma antigens (17) and MUC-1 mucin (18) have been detected by CFC in some cancer patients. The detection of antigen-specific T-cell responses of low frequency depends upon the use of high-affinity antibodies conjugated so as to have very low background staining (19, 20). With such antibodies, as well as good secretion inhibitors (21) and efficient fixation and permeabilization reagents (7), CFC can routinely achieve backgrounds of 0–0.05% cytokine-positive cells in unstimulated whole blood (6–8, 10, 14). Nevertheless, the question arises as to whether this de facto detection limit (−0.05%) is low enough to measure responses to truly tumor-specific antigens, for which the T-cell precursor frequency might be extremely low.

We sought to address this question by measuring the response to a truly tumor-specific antigen, the immunoglobulin idiotype in multiple myeloma, using CFC. Vaccination with

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3 The abbreviations used are: CFC, cytokine flow cytometry; CMV, cytomegalovirus; KLH, keyhole limpet hemocyanin; SEB, staphylococcal enterotoxin B; mAb, monoclonal antibody; IL, interleukin; BrdUrd, bromodeoxyuridine.
expression in myeloma patients, who have a high tumor burden. In proliferation assays, it remains to be determined which assay will provide better predictive power for clinical outcome. Although it is unknown whether CFC will provide such a clinically predictive assay, we show here that CD4 T-cell responses to tumor immunoglobulin, as well as to the KLH carrier protein, are detectable by CFC in some myeloma patients after dendritic cell vaccination. This is despite the presence of considerable immunosuppression in myeloma patients, who have a high tumor burden and have undergone extensive previous chemotherapy. Because there is only partial correlation between CFC and cellular proliferation assays, it remains to be determined which assay will provide better predictive power for clinical outcome.

Materials and Methods

Patients. The nine patients studied were diagnosed with multiple myeloma and consisted of eight males and one female, ages 44-64 (median, 51), seven with IgGk and two with IgAk myeloma proteins. All patients were pretreated with chemotherapy consisting of at least three to six cycles of vincristine, Adriamycin, and dexamethasone. Patients 2 and 5 also received two cycles of cyclophosphamide; patients 3 and 6 also received 1 and 10 cycles, respectively, of vincristine, melphalan, cyclophosphamide, and prednisone; and patient 6 also received three cycles of pulsed dexamethasone. Patients 1, 2, 4, 5, 6, and 7 were tested by CFC and proliferation assays, then vaccinated with autologous dendritic cells pulsed with KLH-conjugated tumor immunoglobulin, and tested again by CFC beginning only after the fourth protein boost. Patients 3, 8, and 9 underwent autologous peripheral stem cell transplantation and were then vaccinated with dendritic cells pulsed with tumor immunoglobulin + KLH beginning 6 months after transplantation. They received a second identical dendritic cell vaccine 1 month later and then five monthly boosts with tumor immunoglobulin + KLH protein. CFC and proliferation assays were performed after each boost, but patients 8 and 9 were analyzed by CFC beginning only after the fourth protein boost.

CFC Assays. Assays were performed by the method of Nomura et al. (7), shown schematically in Fig. 1. Briefly, patient blood was collected in sodium heparin tubes and aliquoted into 15-ml conical polypropylene tubes (Falcon; BD Biosciences, Labware Division, Bedford, MA), 1 ml/tube. Costimulatory mAbs to CD28 and CD49d (BD Biosciences, Immunocytometry Systems, San Jose, CA) were added at a final concentration of 1 μg/ml. Antigen [KLH (Calbiochem, San Diego, CA), tumor immunoglobulin, irrelevant immunoglobulin, SEB (Sigma Chemical Co., St. Louis, MO), or no antigen] was added at a final concentration of 50 μg/ml (0.5 μg/ml for SEB). Samples were incubated at 37°C overnight (~16 h). For some experiments, replicate samples were incubated only 2 h instead of overnight. Brefeldin A (Sigma) was then added at a final concentration of 10 μg/ml, and incubation continued for an additional 4 h. The reaction was then stopped, and adherent cells were removed by the addition of 2 mM (final concentration) EDTA for ~15 min at room temperature, followed by vortexing and addition of 10 ml of BD FACS Lysing Solution (BD Immunocytometry Systems). After 10 min at room temperature, the samples were spun at 500 × g for 10 min, resuspended in 1 ml of freezing media (1% BSA and 10% DMSO in PBS), and frozen at −80°C for later analysis. Upon thawing, cells were

<table>
<thead>
<tr>
<th>Feature</th>
<th>CFC</th>
<th>ELISPOT*</th>
<th>MHC tetramer staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of detection</td>
<td>1:5000</td>
<td>1:30,000–1:300,000</td>
<td>1:10,000–1:50,000</td>
</tr>
<tr>
<td>Turnaround time</td>
<td>8 h</td>
<td>24-48 h</td>
<td>2 h</td>
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<tr>
<td>Compatible with whole blood</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Functional readout (e.g., cytokine production)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Non-MHC restricted</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Multiparameter readout (for T-cell subsets and others)</td>
<td>Yes</td>
<td>No, or more difficult to make</td>
<td>Yes</td>
</tr>
<tr>
<td>Easily automated</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Enzyme-linked immunospot.
Cytokine Flow Cytometry for Tumor Response Detection

**Table 2** Summary of CFC results of myeloma patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor Immunoglobulin</th>
<th>KLH</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>TNF-α</td>
<td>0.28%</td>
<td>0.13%</td>
<td>0.35%</td>
<td>0.10%</td>
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<td>1</td>
<td>0.00%</td>
<td>0.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.00%</td>
<td>0.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.11%</td>
<td>0.35%</td>
<td>0.01%</td>
<td>0.03%</td>
<td>0.06%</td>
<td>0.06%</td>
</tr>
<tr>
<td>4</td>
<td>0.00%</td>
<td>0.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>6</td>
<td>0.01%</td>
<td>0.00%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.06%</td>
</tr>
<tr>
<td>7</td>
<td>0.00%</td>
<td>0.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>ND*</td>
<td>ND</td>
<td>0.00%</td>
<td>0.00%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>ND*</td>
<td>ND</td>
<td>0.01%</td>
<td>0.00%</td>
<td>ND</td>
<td>0.13%</td>
</tr>
</tbody>
</table>

*Results are expressed as net percentage of cytokine-producing CD69+CD4 cells, with background percentage in the absence of antigen subtracted (backgrounds were 0.021 ± 0.017% for IFN-γ and 0.034 ± 0.031% for TNF-α. Numbers in bold, positive responses. Responses were only considered positive if they were ≥0.1% of CD4 cells and >3-fold the background level for that sample.

When multiple postvaccination time points were analyzed, the highest response attained is listed.

Patients 8 and 9 were not analyzed by CFC before vaccination. The responses reported correspond to the following time points: patient 8, after protein boost 4; patient 9, after protein boost 5.

Results

CFC assays (Fig. 1) were performed on nine multiple myeloma patients undergoing vaccination directed against their tumor immunoglobulin idiotype. The myeloma patients received autologous dendritic cells pulsed with tumor immunoglobulin coupled to KLH as a carrier. Samples were taken before vaccination (for seven patients) and after one or more dendritic cell vaccinations and, in some instances, protein boosts. For each time point, individual samples of whole blood were stimulated with no antigen (negative control), SEB (positive control), tumor immunoglobulin, irrelevant immunoglobulin, or KLH. A summary of the patient CFC responses is seen in Table 2.

**Preliminary Results.** Six of the seven patients analyzed before vaccination demonstrated no preexisting response to either KLH or tumor immunoglobulin. One patient had a prevaccination CFC response to tumor immunoglobulin (but not KLH), which was also reflected in a positive proliferation assay to tumor immunoglobulin (but not KLH). In the other six patients, prevaccination responses to either tumor immunoglobulin or KLH were never higher than 0.03% for IFN-γ and 0.05% for TNF-α.

**Development of Responses to Vaccination.** One of the nine patients (no. 1) developed a response to both tumor immunoglobulin and KLH, after only one dendritic cell vaccination (Fig. 2). This response was not idiotype specific, however, because the response to irrelevant immunoglobulin was also positive after vaccination in this patient (data not shown). This was the only patient that developed a positive response to irrelevant immunoglobulin; all other patients were consistently negative. Responses to tumor immunoglobulin or KLH were not detected by proliferation assays in patient 1.

Patient 3 demonstrated a prevaccine CFC and proliferative response to tumor immunoglobulin but not KLH. This response was not durable, because it was no longer detected at later time points. A KLH response developed after the second protein boost in this patient; the response was higher after the fourth protein boost and was also seen by proliferation assay. The CFC KLH response in patient 3 was limited to TNF-α-producing cells (not IFN-γ-producing cells). All other patients’ responses...
to either KLH or immunoglobulin were detectable with both cytokines.

A third patient (no. 9) developed a positive CFC response to KLH, but not tumor immunoglobulin, after the fifth and final protein boost. This patient was also positive by proliferative assay for KLH. Finally, a fourth patient (no. 7) showed an increased frequency of KLH-responsive cells after vaccination (from 0.00% to 0.07% for IFN-γ and 0.00% to 0.06% for TNF-α), but this frequency was below the 0.1% cutoff level established for positive responses. This patient also had a detectable response to KLH using the proliferation assay.

Comparison of Responses to Cancer Vaccines versus Viral Antigens. The positive CFC responses to tumor immunoglobulin and KLH were all in the range of 0.1–0.35% of CD4 T cells for both TNF-α and IFN-γ. This level of response is similar to that typically seen for viral antigens such as mumps and HIV (in long-term nonprogressors) but lower than that seen for CMV (Fig. 3). CMV may represent a special class of viral antigen, because CMV can reside in lymphoid organs and is subject to periodic reactivation, allowing for frequent boosting of T-cell responses in chronically infected individuals. With the exception of CMV, however, the CFC responses observed to cancer antigens in this study are of the same order of magnitude as those observed previously in healthy individuals responding to viral antigens.

Correlation of Responses with Proliferation Assays. To assess the correlation of CFC responses with proliferation assays, we plotted patients’ net responses for each assay and each time point measured. For KLH responses (Fig. 4), there was a positive, although imperfect, correlation between the two assays ($r^2 = 0.25$, $P < 0.05$). For tumor immunoglobulin responses, no significant positive correlation was obtained (data not shown). In part this may be because of the paucity of positive data points observed with tumor immunoglobulin, which is a weaker antigen than KLH.

SEB Responses in Chemotherapy-treated Myeloma Patients. One of the features that we built into the CFC assays was the use of SEB as a positive control. In addition to providing a control for the activation and staining of each sample set, the level of each patient’s response to SEB could be taken as a measure of their global immunocompetence, which may change over time with immunosuppressive therapy and changes in tumor burden. We compared the mean SEB response (from several time points) of the nine myeloma patients in this study with the mean SEB response of five healthy individuals, also measured at several time points. When compared with SEB responses of healthy individuals, the responses of the myeloma patients were significantly lower ($P < 0.02$; Fig. 5). Six of the nine myeloma patients had mean SEB responses below that of the lowest healthy control. Thus, the ability to mount specific immune responses to vaccination in these individuals might be significantly impaired. Nevertheless, we were able to demonstrate the development of anti-immunoglobulin and/or anti-KLH CD4 T-cell responses in three of the nine patients studied; a fourth demonstrated an increase in cytokine-producing CD4 T cells to KLH stimulation that was below the 0.1% cutoff for a positive response. Interestingly, these four patients had the highest mean IFN-γ responses to SEB as well (3.18–5.52%). This suggests a possible correlation between overall immunocompetence, as measured by SEB response, and ability to mount a response to a specific vaccine. Of course, some of the patients may also develop vaccine responses after further courses of vaccination.
immunocompetence in terms of their ability to respond to SEB. Many of the myeloma patients demonstrated reduced overall cytokine production (see Fig. 3), but the use of CD69 facilitates clustering of recently activated cells to 4°C after 6 h of stimulation, allowing for further processing the next day (7).

The responses observed (at 0.1–0.35% of CD4 cells) are at a level that may prove to be immunologically important, because they are in a similar range as many antiviral responses observed with CFC assays (10, 13). This is despite the fact that many of the myeloma patients demonstrated reduced overall immunocompetence in terms of their ability to respond to SEB.

We chose to analyze two cytokines, TNF-α and IFN-γ, based upon our previous data that CD4 cells producing these cytokines are more frequent in humans than CD4 cells producing IL-2, IL-4, IL-5, or other T helper 2 cytokines. This is true not only for viral antigens but for mitogen responses as well (19, 32). Although it is possible that cancer patients may display a different cytokine bias, this is not the case for HIV-specific responses in HIV disease, where TNF-α and IFN-γ still predominate (12–14). All but one myeloma patient that demonstrated a response with one cytokine was also positive for the other, and the frequency of positive cells for each cytokine was usually similar. However, patient 3 developed a KLH response that was restricted to TNF-α-producing cells, as measured at two separate time points after vaccination. Thus, TNF-α production could be seen in the absence of IFN-γ production but not vice versa. It is possible that the combined production of IFN-γ and TNF-α by antigen-specific T cells represents a stronger bias toward T helper 1 immunity and may correlate with stronger cytotoxic responses as well.

CD69, an early activation marker, was used in combination with cytokines to identify cells that had been activated by the in viv o antigen restimulation. Most cytokine-positive cells are also CD69 + (see Fig. 3), but the use of CD69 facilitates clustering of the responding cells in a two-dimensional space and ensures that the cytokine signal is derived from activated cells.

The present study used a 20-h stimulation time, the last 4 h in the presence of Brefeldin A, for all CFC assays presented. This time was chosen for convenience to accommodate clinical samples arriving late in the day, by incubating all samples overnight prior to addition of Brefeldin A. Other studies using CFC assays have demonstrated that 6 h of stimulation is optimal for most cytokines in the CMV system (7). When several cancer patient samples were compared in a 6-h versus overnight assay, no consistent trend in results was observed. However, it is possible that some responses may have been detected more readily at 6 h. It would also be practical to achieve 6-h stimulation by the use of a timed cooling device that chills the activated cells to 4°C after 6 h of stimulation, allowing for further processing the next day (7).

Positive CFC responses to KLH or tumor immunoglobulin
were observed in six different assays in three patients. Four of these six assays correlated with positive proliferation responses for the same samples. However, there were several proliferative responses observed in samples that were not positive by CFC, and two CFC responses (both in the same patient) that were not detected as positive by proliferation assay. Overall, the correlation of proliferative and CFC assays for KLH was significant (P < 0.05) but imprecise (r² = 0.25), whereas the same correlation for tumor immunoglobulin did not reach statistical significance. Because the proliferation assay does not distinguish between different T-cell subsets, it is possible that some degree of discordance may be attributable to proliferation by non-CD4 cells (e.g., CD8 and natural killer cells). One remedy for this would be the use of BrdUrd as a thymidine analogue for proliferation assays. By using fluorescent conjugates of anti-BrdUrd antibody and DNase treatment to gain access to the cell nucleus, BrdUrd-positive cells can be detected by multiparameter flow cytometry, with simultaneous staining for CD4 or other markers (33). A significant positive correlation between CFC and BrdUrd assays has been reported for CD4 cells responding to SEB (33) and HIV.4

The present study suggests that CFC assays are capable of detecting responses to current-generation vaccines in at least some patients. There is a significant discordance between traditional proliferation assays and CFC. However, proliferation assays are already known to be an imperfect predictor of clinical outcome; therefore, substitute assays should not be expected to correlate completely with proliferation assays. Rather, new assays should be chosen as substitutes if they are better predictors of clinical response. Other immunological factors (such as CD8 T-cell response, antibody response, and others), as well as nonimmunological factors, may affect clinical status. However, it is possible that the central role of CD4 cells in both cellular and humoral immunity makes the measurement of CD4 responses particularly significant as a single-factor indication of the immune response. Longer term immunological and clinical follow-up of the patients in this study, as well as additional patients, will be necessary to determine whether CFC for CD4 cells provides a useful surrogate of effective immunization in cancer patients.

Acknowledgments

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References


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