Detecting T-cell reactivity to whole cell vaccines
Proof of concept analysis of T-cell response to K562 cell antigens in CML patients

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Keywords: chronic myeloid leukemia, cross-presentation, GM-CSF, immune monitoring, K562, whole cell vaccine

Introduction

Chronic myeloid leukemia (CML) is characterized by the presence of the BCR-ABL translocation,1 and is known to be particularly immunosensitive. The high susceptibility of CML to immune destruction has been evidenced by the relatively high rates of durable remission achieved following hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI).2 However, the utility of these treatments is often undermined by the risks of severe toxicity, most commonly in the form of acute or chronic graft-versus-host disease (GVHD). To overcome these limitations, therapeutic cancer vaccines for CML have been developed for the specific activation of the immune system against CML cells.3,7 Whole tumor cell-based vaccination has emerged as a promising strategy to treat a variety of malignancies,8-12 and has the advantage—over peptide-based approaches—of providing a range of antigens for the stimulation of polyclonal immune responses. In particular, the BCR-ABL+ K562 cell line has been shown to be amenable to large-scale manufacture, and has been developed as a clinical grade vaccine reagent.13,14

To determine the efficacy of cancer vaccines, methods are needed to monitor the development of antitumor immunity. This can readily be accomplished with peptide-based vaccines, as the immunizing epitopes are known. In contrast, whole tumor cell-based vaccines by definition comprise a complex mixture of antigens, and those specifically targeted by therapy-induced immunity are unknown. An attractive approach to this challenge is to employ whole tumor cells themselves as an antigen source, enabling the screening of all tumor antigens to which a patient was exposed. On the other hand, whole tumor cells in their natural state are often poorly immunogenic, and may only weakly stimulate an immune response.

One strategy for displaying antigens derived from whole tumor cells is through their cross-presentation by antigen-presenting cells (APCs), such as dendritic cells (DCs). Apoptotic bodies can be efficiently ingested, processed and their antigens cross-presented on MHC Class I molecules by immature dendritic cells (iDCs), leading to T-cell activation.15,16 This cross-strategy has previously been developed for DC-based vaccines.17-19 Here, we describe the optimization and application of a K562 cell-based assay to detect CML-associated immunity following the administration of an effective immunotherapy for CML, DLI, as well as following K562 cell-based vaccination. Our studies demonstrate that developing a whole cell-based...
A disease-specific assay that can be readily applied to the monitoring of cell-based cancer vaccines is feasible.

Results

Immature DCs effectively phagocytose apoptotic K562 cells. Several studies have identified that apoptosis results in efficient antigen cross-presentation to T cells.\(^1\) We sought to adapt this concept for developing an assay to monitor the immunologic activity of K562 cell-based tumor vaccines. As schematically represented in Figure 1, our workflow was to first optimize conditions for K562 cell death and for phagocytosis by DCs. We then wanted to ensure that cellular antigens derived from apoptotic K562 cells could be cross-presented to stimulate antigen-specific T-cell responses. To this aim, we tested our assay first for the detection of anti-viral responses against a known immunogenic viral antigen introduced into K562 cells, and second, for the detection of CML-specific immunity in CML patients successfully treated with DLI. Finally, we applied our assay to peripheral blood mononuclear cells (PBMCs) collected from CML patients who had been vaccinated with an experimental antigen cross-presentation to T cells.\(^1\) We sought to adapt this procedure, donor lymphocytes are infused into patients, typically in the absence of further chemo- or radio-therapy, leading to durable remission in 75–80% patients.\(^1\) Numerous reports have described the generation of CML antigen-specific antibodies and CD8+ T-cell responses following DLI.\(^1\) We therefore examined this group of patients for evidence of increased anti-K562 T-cell reactivity.

Figure 1. Schema for developing a cross-presentation assay for measuring immunoreactivity to K562 cells.
immunity developing upon DLI. We selected PBMC samples from 4 DLI-responsive patients (Patients DLI1–4), collected before and after DLI (at the time of clinical response) (Table 1). This patient group was clinically homogeneous: all patients relapsed 6–15 months following myeloablative allogeneic HSCT, received CD8-depleted donor lymphocytes for the treatment of relapsed stable-phase CML, and promptly developed cytogenetic and molecular responses (median 2.75 and 8 months post-DLI, respectively).

As shown in Figure 4B, we observed increased CD8+ T-cell reactivity to cross-presented apoptotic K562 cells in a DLI-responsive patient via ELIspot assays (average 16.5 spots/100,000 cells), compared with samples from the same patient prior to DLI (average 0 spots/100,000 cells), (p = 0.08). Furthermore, we detected only minimal reactivity in three CML patients who had been treated with non-immunologic therapy (imatinib, 800 mg daily for 1–7 y; Table 2). Our results are consistent with the detection of CML-specific T cells following successful immunotherapy for CML, and suggest the potential utility of this assay for detecting CML specific T-cell responses following other forms of immunotherapy for CML.

GM-K562 vaccination elicits a T-cell response against CML antigens. At Dana-Farber Cancer Institute (DFCI), we launched a clinical trial to test the effects of whole tumor cell-based vaccines in patients with evidence of persistent minimal residual disease following treatment with imatinib. The vaccine consisted of lethally irradiated K562 cells (as a source of CML antigen) genetically modified to express GM-CSF (as adjuvant), and was administered as a series of 9 subcutaneous/intradermal injections. These vaccines were well-tolerated, although transient skin reactions at the site of injection were common (Fig. 5). To identify possible responses to all commonly expressed CML antigens, we screened patients for CD8+ T-cell immune responses against K562 cells.

We analyzed PBMC samples collected from three patients (Patients A–C) who were vaccinated with the GM-CSF expressing K562 vaccine (Table 3). We focused our analysis of CD8+ T-cell reactivity to apoptotic/cross-presented K562 cells at three time points: approximately 30 days prior to vaccination; at the middle of the vaccination cycle (vaccine #5, or day 50); and 30 days after the final vaccine (~day 170). We tested CD8+ T cells against autologous DCs that were fed apoptotic K562 cells on ELIspot assays, compared with apoptotic K562 cells or autologous DCs alone. Results of the ELIspot assays were integrated with counts of absolute white blood cell (WBC) numbers, molecular detection of tumor burden, and immunophenotype of peripheral blood T cells throughout the vaccination period (Fig. 6).

For both Patients A and B, transiently increased anti-CML T-cell reactivity was detected at mid-vaccination cycle, while Patient C demonstrated an absence of reactivity at all time points. Patient A exhibited increased anti-K562 antigen reactivity from 101 spots/100,000 cells prior to vaccination, to 154.5 spots/100,000 cells following the 5th vaccine, that then
promising. Whole cell-based vaccines can be autologous or allogeneic, and may be modified (e.g., to express immunomodulatory molecules), fused with DCs, or unmodified (e.g., in the form of lysates or irradiated whole cells). The promise of this vaccine formulation has been primarily attributed to the multi-epitope nature of the immunogen. However, this same feature also creates challenges for monitoring, since the stimulating antigen(s) are unknown. One commonly employed monitoring approach uses candidate tumor-associated antigens as representative targets for immune responses. For example, in the case of CML, BCR-ABL, proteinase 3 and Wilms tumor 1 (WT1) all are highly expressed and the latter two are known to be immunogenic.

Figure 4. Apoptotic K562 cells when presented on DCs can stimulate antigen-specific immunity against either introduced or native antigen. (A) K562 cells were transfected to express M1 (see inset) and then irradiated with 400 mJ UVB to induce apoptosis. Following irradiation, these cells were co-cultured with iDCs for 24 h and matured using a cytokine cocktail for 24 h. On ELISPOT, we used CD8+ T cells alone as a negative control, M1-pulsed DCs and PHA as positive controls, and apoptotic M1-K562 and DCs alone on CD8+ T cells to establish background reactivity. There was a significant increase in reactivity of apoptotic M1-K562 when cross-presented by DCs compared with apoptotic M1-K562 cells alone (*p = 0.04) or DC cross-presented apoptotic K562 cells (p = 0.04). Significance was determined using the two-tailed Student’s t-test. All experiments were performed in duplicate wells, and mean spot-forming cells per 100,000 CD8+ T cells are reported. Error bars represent two standard deviations. The ELISPOT image is representative of more than 3 experiments, while the bar graph displays average results from four experiments, with standard deviation as shown. (B) T cells from pre- and post-DLI patients were tested on ELISPOT against CML antigens in the form of apoptotic K562 cross-presented on DCs, and results were reported as spots per 100,000 CD8+ T cells (minus background, defined as reactivity of apoptotic K562 cells alone or autologous DCs alone on CD8+ T cells); p = 0.08 (Student’s t-test). Three patients treated with imatinib were tested as comparison, and showed no reactivity. Results are shown as means with error bars representing two standard deviations.

Among multiple approaches for the design of anticancer vaccines, whole cell-based strategies have so far proved to be the most promising. Whole cell-based vaccines can be autologous or allogeneic, and may be modified (e.g., to express immunomodulatory molecules), fused with DCs, or unmodified (e.g., in the form of lysates or irradiated whole cells). The promise of this vaccine formulation has been primarily attributed to the multi-epitope nature of the immunogen. However, this same feature also creates challenges for monitoring, since the stimulating antigen(s) are unknown. One commonly employed monitoring approach uses candidate tumor-associated antigens as representative targets for immune responses. For example, in the case of CML, BCR-ABL, proteinase 3 and Wilms tumor 1 (WT1) all are highly expressed and the latter two are known to be immunogenic. However, these individual antigens may not be consistently immunogenic across patients, and only a subset of patients may bear the restricting HLA-allele associated with known antigen-derived epitopes. This was suggested by Smith et al. since they observed decrement in tumor burden following vaccination of CML patients with irradiated, modified K562 cells and development of humoral immunity against whole tumor-cell lysates, but absence of detectable T-cell immunity against a panel of
known CML associated antigens. Presumably, T-cell reactivity developed against unknown CML antigens.

Our study demonstrates that developing a robust assay to measure T-cell reactivity to leukemia antigens from whole tumor cells is feasible. While other sources of whole-cell tumor antigens for this purpose are available, such as total RNA or tumor lysates, we favored using apoptotic bodies since they have been demonstrated to efficiently provoke T-cell responses. We focused on developing a K562 cell-based assay, since clinical studies at our center and elsewhere have used irradiated K562 cells as a vaccine reagent. These studies include approaches using K562 cells either directly as a therapeutic vaccine for myeloid leukemia or as bystander cells, for cell-based delivery of cytokine adjuvants or other immunologically active molecules. Thus, the development of a whole cell-based assay to detect anti-K562 T-cell responses might enable the screening of T-cell responses to leukemia antigens, for determining the immunogenicity of bystander cells. Finally, by detecting anti-leukemia T-cell reactivity, our assay can provide a stepping-stone for the discovery of novel leukemia antigens, if used in combination with approaches such as T cell based cDNA expression library cloning.

We applied our assay to monitor three CML patients who were exposed to a series of 9 vaccinations with GM-CSF-secreting K562 cells over 20 weeks, and integrated these results with information on molecular tumor burden and T-cell immunophenotype. Despite the limitations of a small sample size, the results of our monitoring studies stimulate further investigation. In two of three patients, we observed a 50% increase in T-cell reactivity to CML antigens during the course of vaccination. This mid-cycle reactivity coincided with 1-log decreases in molecular tumor burden. Patient B further demonstrated a dramatic decrease in ratio of Tregs to effector T cells at this time. While the results for each assay measurement were modest, taken together they suggest that the vaccine has a biologic activity against CML.

Both Patients A and B were further observed to lose anti-K562 reactivity later in the vaccine course. On the one hand, this pattern could reflect the effects of the vaccine administration schedule. All vaccines were initially administered weekly (×3) and then gradually tapered off to bi-monthly (×3), then finally as monthly vaccines (×3). Possibly, a high frequency of vaccination is vital to inducing antitumor responses, and for overcoming endogenous tumor-associated immunosuppression. Alternatively, induction of immunity could also activate counter regulatory mechanisms that function to suppress excessive immune activity, i.e., generation of exhausted and inefficient T cells with continuous antigenic stimulation. Furthermore, GM-CSF itself can function as both an immunostimulatory and immunosuppressive molecule.

In summary, our studies using a K562 cross-presentation assay are suggestive of the development of anti-CML T-cell reactivity following whole leukemia-cell vaccination. Moreover, our results suggest that alternative adjuvants and/or administration schedules might be required to generate more consistent responses. The availability of our K562 cross-presentation assay renders benchmark antitumor immunity measurements possible, from which the effects of further modifications to this promising immunogen formulation can be assessed.

Materials and Methods

Patient samples. Heparinized peripheral blood from normal volunteers and from CML patients were collected during their course of treatment while participating in clinical research protocols at the Dana-Farber Cancer Institute (DFCI), Boston MA. All clinical protocols were approved by the DFCI Human Subjects Protection Committee. The K562-GM-CSF trial also received approval from the Food and Drug Administration and the NIH Recombinant Advisory Committee on Gene Transfer. Peripheral blood mononuclear cells (PBMCs) from normal donors and patients were isolated by Ficoll/Hypaque density-gradient centrifugation (GE Healthcare Bio-Sciences AB), cryopreserved with 10% DMSO, and stored in vapor-phase liquid nitrogen until the time of analysis.

Induction of apoptotic death of K562 cells. The BCR-ABL+ erythroleukemia cell line K562 (ATCC) was cultured in 162 cm² tissue culture flasks (Corning Inc.) from an initial concentration of 1 × 10⁵ cells/mL, using media consisting of RPMI 1640 (Cellgro Mediatech Inc.) with 10% fetal bovine serum (FBS) (Lonza). K562 cells were harvested on day 3 of culture, washed and suspended in PBS (Mediatech Inc.) at a concentration of 5 × 10⁵ cells/mL. 5 × 10⁶ of these cells were plated in PBS in each well of 6-well plates (Corning Inc.), and irradiated with UVB (0 to 2,000 mJ; Stratalinker UV Crosslinker 1800, Stratagene).

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| Table 1. Clinical characteristics of DLI-treated CML patients samples whose PBMC were tested by ELISpot against the K562 cross-presentation assay |
|---|---|---|---|---|---|
| Patient | Age/Sex | Months from HSCT to relapse | Months from HSCT to DLI | Months from DLI to cytogenetic remission | Months from DLI to molecular remission |
| DLI1 | 40/F | 6 | 24 | 3 | 7 |
| DLI2 | 50/M | 14 | 25 | 3 | 8 |
| DLI3 | 59/F | 15 | 14 | 2.5 | 8 |
| DLI4 | 25/F | 11 | 54 | 2 | 12 |

| Table 2. Clinical characteristics of Imatinib treated CML patient samples whose PBMC were tested by ELISpot against the K562 cross-presentation assay |
|---|---|---|---|
| Patient | Age/Sex | Previous therapy | Imatinib maximum dose; length of treatment |
| IM1 | 65/M | Hydroxyurea, interferon, busulfan | 800 mg/daily; 3 y |
| IM2 | 67/M | Hydroxyurea, interferon | 800 mg/daily; 7 y |
| IM3 | 64/M | Hydroxyurea, interferon | 800 mg/daily; 1 y |
Following irradiation, the cells were incubated at 37°C until harvest. Two to 24 h following irradiation, K562 cells were labeled with AnnexinV and propidium iodide (PI) stains, according to the manufacturer’s instructions (BD PharMingen), and analyzed by flow cytometry (Cytomics FC 500, Beckman Coulter Inc.). The extent of apoptosis was defined as the percentage of AnnexinV positive and PI negative cells based on 50,000 events.

Isolation and preparation of monocyte-derived DCs. To generate DCs, frozen PBMCs were thawed and monocytes were selected through immunomagnetic CD14+ bead selection (Miltenyi Biotech). The CD14+ cells were cultured at 2 million cells were selected through immunomagnetic CD14+ bead selection.

Detection of antigen specific T-cell responses. We used the ELISpot assay to measure the frequency of reactive IFNγ secreting CD8+ T cells at 7 d after initiation of in vitro stimulation. Nitrocellulose membrane 96-well plates (Multiscreen, Millipore) were coated with anti-human IFNγ monoclonal antibody (1-D1K; Mabtech). One day prior to plating the antigen-primed T cells on the ELISpot plate together with different test antigens, we selected cytotoxic T cells by immunomagnetic CD8 selection (Miltenyi Biotech). The CD8+ T cells were starved overnight in serum-free AIM-V medium (Gibco) and 1% glutamine (2 mM) to reduce ELISpot background. IFNγ secretion was detected using capture and detection antibodies as directed (Mabtech).

Table 3. Clinical characteristics of GM-CSF secreting irradiated K562 cell treated CML patient samples whose PBMC were tested by ELISpot against the K562 cross-presentation assay

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Previous therapy</th>
<th>Imatinib maximum dose; length of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>42/M</td>
<td>-</td>
<td>800 mg/daily; 2 y</td>
</tr>
<tr>
<td>B</td>
<td>45/M</td>
<td>Hydroxyurea</td>
<td>800 mg/daily; 1 y</td>
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<tr>
<td>C</td>
<td>55/M</td>
<td>Hydroxyurea</td>
<td>800 mg/daily; 5 y</td>
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Figure 5. Skin reactions characterized by erythema, swelling and warmth occurred commonly at the immunization site, following sc/id injection of irradiated GM-CSF secreting K562 cells. These reactions were transient, lasting 1–5 d following vaccination.
AB) and imaged using an ImmunoSpot Series Analyzer (Cellular Technology Ltd.).

**K562 vaccination clinical protocol.** Patients received a series of 9 vaccinations on a clinical trial conducted at DFCI from 2005 to 2008 (registered as NCT00301093 on www.clinicaltrials.gov). Each vaccine contained $5 \times 10^6$ GM-K562 cells that had been lethally irradiated with 10,000 cGy γ-rays. Vaccines were administered once a week ($\pm 1$ d) for three weeks, then every other week ($\pm 1$ d) for three doses and then every 28 d ($\pm 2$ d) for three doses. Peripheral blood samples were collected monthly from study subjects for molecular BCR-ABL monitoring (Department of Molecular Diagnostics, Brigham and Women’s Hospital) and immunophenotyping for the first nine months after initiation of vaccines. The monoclonal antibodies used for immunophenotyping were directed against CD8 (clone B9.11; Beckman-Coulter), CD4 (clone 13B8.2; Beckman-Coulter), FOXP3 (clone PCH101; eBioscience), and CD25 (clone B1.49.9; Beckman-Coulter).

**Statistical analyses.** Two-tailed Student’s t-test with pooled variance were applied to determine the difference between groups. p values < 0.05 were considered significant.

**Disclosure of Potential Conflicts of Interest**
The authors declare no competing financial interests.

**Acknowledgments**
The authors would like to acknowledge Nathalie Blachere and Nir Hacohen for their insightful discussions in the development of this research. We thank Mr Quinlan L. Sievers for excellent technical assistance. We would like to acknowledge the generous support from the Pasquarello Tissue Bank, and from the clinical transplant

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**Figure 6.** Immunologic monitoring of CML patients subjected to therapeutic vaccination with irradiated GM-CSF-secreting K562 cells. Reactivity to K562 antigens by cross-presentation assay (measured by ELISpot and shown on bottom row) in relation to: timing of vaccination (indicated by arrows, top row); to absolute WBC count (top row); molecular response, measured by % BCR-ABL/GUS transcript in PBMNC (mid row); and ratio of CD8+ cells to regulatory T cells (Tregs) (defined as FOXP3+/CD25+), measured by flow cytometry (bottom row).
teams at the DFCI, Boston. We also acknowledge support from the Department of Defense for this project. C.J.W. acknowledges support from the Department of Defense (W81XWH-07-1-0080), the Miles and Eleanor Shore Award, NCI (5R21CA115043-2), the Early Career Physician-Scientist Award of the Howard Hughes Medical Institute, and is a Damon-Runyon Cancer Research Foundation Clinical Investigator (CI-38-07).

A.B. and C.J.W. designed the study, performed research, analyzed data, and wrote the manuscript. U.H., M.S., and T.S. performed research. J.S.L. and R.M. generated the clinical grade GM-K562 vaccination reagent. D.N. provided statistical analysis for the vaccine trial and this study. M.W., C.C., J.R., G.D. and D.D. conducted the clinical trial and provided critical patient samples. All authors edited the paper.

References


