### Detecting T-cell Reactivity to Whole Cell Vaccines

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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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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 (H SCT) 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 therapy to treat a variety of malignancies,8-12 and has the advantage—over peptide-based approaches—of providing a range of 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...
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.\(^{15,16,18}\) 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 anti-CML immunotherapy, namely lethally irradiated K562 cells modified to secrete granulocyte macrophage colony-stimulating factor (GM-CSF) adjuvant.

Early apoptosis, defined as the stage at which cells are AnnexinV\(^+\) (have exposed phosphatidylserine on the cell surface) and PI\(^-\) (have intact plasma membranes), has been identified as an optimal stage the uptake of apoptotic cells by DCs.\(^{19,21}\) We selected UVB as the method of apoptosis, as this is an established, inexpensive and simple approach.\(^{17,18,21,22}\) We investigated a range of intensities (50–2,000 mJ) and followed cells over a period of 2 to 48 h (Fig. 2A). Intensities ranging from 300 to 500 mJ consistently produced a high rate of early K562 apoptosis, which was not seen at lower intensities. Intensities higher than 500 mJ caused rapid necrosis. Irradiation with 400 mJ consistently resulted in approximately 35–45% early apoptosis, and 65–75% total apoptosis (AnnexinV\(^+\)PI\(^-\)) at 15 h post exposure (Fig. 2B). We thus selected these energies and time points for inducing apoptosis of K562 cells in subsequent experiments.

To ensure that iDCs are capable of taking up apoptotic K562 cells, iDCs from normal donors were stained with the green fluorescent dye PKH26. These labeled iDCs were then co-cultured with K562 cells that had been labeled with a red fluorescent dye (PKH26) for 24 h at ratios of 1:1, 1:2, 1:5 and 1:9. A higher ratio of K562 cells to iDCs generally resulted in higher rates of phagocytosis. High levels of phagocytosis of approximately 80–85% (visualized as double positive cells on flow cytometry) were observed at physiological temperature (Fig. 3). On the other hand, minimal phagocytosis was observed when the same process was performed at 4°C.

**Cross-presenting DCs effectively stimulate antigen-specific T cells.** We next examined whether apoptotic K562 cells can be effectively processed and cross-presented by DCs to stimulate antigen-specific T cells. For these experiments, we used the well-characterized HLA-A*0201 restricted and immunodominant influenza M1 epitope as a model antigen.\(^{18,23,24}\) We introduced the influenza matrix protein into K562 cells through DNA plasmid nucleofection. We reasoned that if we could induce expression of this protein (and consequently of the M1 epitope), detection of M1-specific CD8\(^+\) T-cell immunity from normal HLA-A2\(^+\) volunteers by ELISpot assay would confirm that cross-presentation of K562 antigens had taken place.

As shown in Figure 4A (inset), we could achieve expression of influenza matrix protein in K562 cells (M1-K562) by nucleofection. We then induced early apoptosis of M1-expressing K562 cells using the conditions described above. These apoptotic cells were fed to HLA-A2\(^+\) DCs isolated from normal adult volunteers, which were then used to stimulate autologous CD8\(^+\) T cells. As expected, we found that DCs fed with apoptotic M1-K562 cells could induce M1-specific CD8\(^+\) T-cell responses (average 5.25 spots/100,000 T cells; p = 0.04) (Fig. 4A). In contrast, apoptotic K562 cells or non-cross-presented M1-K562 cells alone (average 1.5 spots and 0.5 spots/100,000 cells, respectively), or DCs fed with apoptotic K562 cells lacking expression of the influenza matrix protein (0.75 spots/100,000 cells) generated only minimal reactivity. These results demonstrate that the cross-presentation of antigens by DCs can effectively be used to detect T-cell reactivity.

**CML-specific reactivity can be detected following anti-CML immunotherapy.** DLI is a highly effective immunotherapy for the treatment of transplanted patients with relapsed CML. In 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.\(^{25}\) Numerous reports have described the generation of CML antigen-specific antibodies and CD8\(^+\) T-cell responses following DLI.\(^{26-28}\) We therefore examined this group of patients for evidence of increased anti-K562 T-cell

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**Figure 1. Schema for developing a cross-presentation assay for measuring immunoreactivity to K562 cells.**

- **Optimization**: Identification of appropriate conditions for K562 cells to:
  - Apoptosis
  - Phagocytosis
- **Validation**: Detection of T cell responses against:
  - A known immunodominant viral antigen
  - CML patients
- **Application**: Monitoring of T cell responses in patients treated with:
  - CML vaccines using whole K562 cells
  - Vaccines using K562 as bystander 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
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

decremented to 82.5 spots/100,000 cells at day 170. Likewise, T cells in Patient B revealed increased reactivity from 12.5 spots/100,000 cells prior to vaccination to 25 spots/100,000 cells following the 5th vaccine, which then declined to 13.5 spots/100,000 cells at day 170. None of the patients demonstrated alterations in their circulating WBCs. For Patients A and B, however, concomitant decrements of approximately one log were observed in molecular tumor burden (%BCR-ABL/GUS transcript). At the same time, an increased ratio of effector CD8+ to regulatory T cells (Tregs) was observed in Patient B. As for Patient C, the absence of T-cell reactivity was associated with a gradual one log increase in molecular tumor burden over the 170 days. Of note, both Patients A and B subsequently demonstrated prolonged stable disease on drug therapy despite persistent minimal residual disease, while Patient C, with rising tumor burden, subsequently underwent HSCT.

**Discussion**

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.

### Materials and Methods

**Patient samples.** Heparinized peripheral blood from normal volunteers and from CML patients were collected during their course of treatment while participating on 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 cm\(^2\) tissue culture flasks (Corning Inc.) from an initial concentration of 1 × 10\(^5\) 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 × 10\(^5\) cells/mL. 5 × 10\(^6\) 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).
Additional quantities of GM-CSF (120 ng/mL) and IL-4 (70 ng/mL) were added on days 3 and 5 to generate iDCs. DCs were selected through immunomagnetic CD14+ bead selection (Miltenyi Biotech). The CD14+ cells were cultured at 2 million cells per well, and maintained in Iscove’s modified Dulbecco’s media (IMDM) supplemented with 20% FBS, 1% glutamine (2 mM), penicillin (50 IU/mL; Mediatech Inc.) and streptomycin (50 μg/mL; Mediatech Inc.).

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 in 3 mL of RPMI supplemented with 2% non-HI FCS (Cellgro), 2 mM glutamine, 50 μg/mL human transferrin (Roche), 5 μg/mL human insulin (Sigma-Aldrich), and 15 μg/mL gentamycin (Invitrogen) in the presence of 120 ng/mL GM-CSF (R&D Systems) and 70 ng/mL IL-4 (R&D Systems) in 6-well plates. Additional quantities of GM-CSF (120 ng/mL) and IL-4 (70 ng/mL) were added on days 3 and 5 to generate iDCs. DCs were matured on day 7 by addition of a cocktail consisting of GM-CSF (50 ng/mL), IL-4 (20 ng/mL), tumor necrosis factorα (TNFα; 10 ng/mL) and prostaglandin E-2 (PGE-2; 1 μM) (all from R&D Systems). We confirmed that DCs were consistently immature at day 6 and mature at day 8 using flow cytometric analysis with the markers CD80, CD86, HLA-DR and CD83.

Uptake of apoptotic bodies by antigen-presenting dendritic cells. For most experiments, apoptotic K562 cells served as source of antigenic ‘food’ for dendritic cells. Day 6 iDCs were co-cultured together with irradiated K562 cells at a 1:9 ratio at 37°C in DC media. After 24 h, fresh DC media containing the DC maturation cytokine cocktail was added to the co-culture, and incubated for a further 24 h. For some experiments, we introduced the influenza matrix protein into K562 cells by DNA nucleofection (Nucleofector II, Amaza Biosystems) of a plasmid encoding the influenza matrix protein (gift of Matthew Albert, Rockefeller University, NY) on day 2 of culture to create M1-expressing K562 cells (M1-K562). M1-K562 cells were cultured overnight before apoptosis induction by UVB irradiation on day 3.

To confirm phagocytosis of apoptotic K562 cells, we stained K562 cells red with PKH26 (Sigma-Aldrich), according to the manufacturer’s instructions, prior to induction of apoptosis. iDCs were stained green with PKH2 (Sigma-Aldrich), according to the manufacturer’s instructions, following harvest on day 6 and immediately prior to co-culture of the two cell populations. The two stained cell populations were combined immediately following K562 irradiation in a 1:9 ratio and co-cultured in DC media for 24 h at 37°C (to encourage phagocytosis) or 4°C (to inhibit phagocytosis). Phagocytosis of apoptotic bodies by DCs was defined by the percentage of cells that were double positive for both PHK2 and PKH26 stains on flow cytometry.

In vitro expansion of antigen-specific T cells. To expand autologous K562 reactive T cells, we thawed autologous PBMCs and stimulated them for 7 days by co-culture with mature DCs that had been fed apoptotic K562 cells in T-cell media in 24-well plates (Becton Dickinson). 2–3 × 10^5 PBMCs were placed in each well and maintained in Iscove’s modified Dulbecco’s media (IMDM) (Cellgro Mediatech Inc.) that was supplemented with 20% FBS, 1% glutamine (2 mM), penicillin (50 IU/mL; Mediatech Inc.) and streptomycin (50 μg/mL; Mediatech Inc.). IL-7 (10 ng/mL) was added on day 0 and 4; IL-2 (50 U/mL) (both R&D Systems) was also added on day 4. For some experiments, T cells were expanded against peptide-pulsed controls. DCs were generated as described and pulsed with M1 peptide (10 μg/mL) for 2 h prior to co-culture with autologous PBMCs for 7 days.

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 (Multiscreeen, 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)
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 $\gamma$-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.

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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 PBMC (mid row); and ratio of CD8+ cells to regulatory T cells (Tregs) (defined as FOXP3+/CD25+), measured by flow cytometry (bottom row).
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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-KS62 vaccination reagent. D.N. provided statistical analysis for the vaccine trial and this study. M.W., R.S., C.C., J.R., G.D.D. and T.S. conducted the clinical trial and provided critical patient samples. All authors edited the paper.


