Microenvironment and Immunology

Efficacious Immune Therapy in Chronic Myelogenous Leukemia (CML) Recognizes Antigens That Are Expressed on CML Progenitor Cells

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Abstract

Curative effects of graft-versus-leukemia–based therapies such as donor lymphocyte infusion (DLI) for chronic myelogenous leukemia (CML) may result from immunologic ablation of self-renewing CML progenitor cells. Patients who achieved durable remissions after DLI developed a significant B-cell lymphocytosis after treatment, which did not occur in patients who were unresponsive to DLI. In this study, we identified antigen targets of this B-cell response by probing two immunoproteomic platforms with plasma immunoglobulins from seven CML patients with clinically apparent graft-versus-leukemia responses after DLI. In total, 62 antigens elicited greater reactivity from post-DLI versus pre-DLI plasma. Microarray analysis revealed that >70% of the antigens were expressed in CML CD34+ cells, suggesting that expression in malignant progenitor cells is a feature common to antibody targets of DLI. We confirmed elevated expression of three target antigens (RAB38, TBCE, and DUSP12) in CML that together consistently elicited antibody responses in 18 of 21 of an additional cohort of CML patients with therapeutic responses, but not in normal donors and rarely in non-CML patients. In summary, immunologic targets of curative DLI responses include multiple antigens on CML progenitor cells, identifying them as potential immunogens for vaccination and/or monitoring of immunotherapeutics designed to eliminate myeloid leukemia stem cells. Cancer Res; 70(3): 906–15. ©2010 AACR.

Introduction

The elimination of minimal residual disease remains a critical issue in the treatment of patients with chronic myelogenous leukemia (CML). Immune-based therapies for CML such as allogeneic hematopoietic stem cell transplantation (HSCT) can produce durable remissions associated with molecularly undetectable disease. The curative effect of HSCT results from immunologic recognition of malignant host cells by donor-derived cells, called the graft-versus-leukemia (GVL) response (1). Donor lymphocyte infusion (DLI) for the treatment of relapsed CML strikingly illustrates the potent antitumor effects of GVL responses. In this therapy, donor mononuclear cells are infused after allogeneic HSCT in the absence of further chemotherapy or radiotherapy. Durable disease remission is achieved in 75% to 80% of patients with relapsed CML treated with DLI (2). Because the curative effect of DLI for CML must result from the elimination of the CML progenitor cell, we hypothesize that its immunologic targets must include antigens expressed on the leukemic progenitor cell. In support of this hypothesis, our recent studies show a progenitor-specific expression pattern for two previously identified DLI-associated antigens: CML66 and CML28 (3).

We previously noted that clinical responses temporally correlated with the development of new potent humoral immunity against antigens with selective expression in malignant cells implicating a key role for B cells in effective DLI responses (4). In the current study, we broadened our panel to 62 DLI-associated antigens by screening two complementary immunoproteomic platforms, a CML expression library and a high-density protein microarray, using plasma from seven patients with comparable clinical courses. All patients received DLI for relapsed CML and responded without graft-versus-host disease (GVHD). The consistent clinical features and robust antitumor responses among this group of patients enabled us to uncover common features of the immunologic targets of DLI and thus gain insight into the mechanisms underlying GVL. Analysis of the gene expression patterns of these antigens in established data sets of normal and malignant hematopoietic tissue confirmed that >70% of these antigens are expressed in CML progenitor cells, supporting our hypothesis that DLI targets CML progenitor cells.
Additionally, we found that several of the identified antigens are selectively expressed in malignant CD34+ cells compared with their normal equivalents. As a collection, these antigens potentially represent highly informative reagents for immunologic monitoring of patients treated with therapies designed to eliminate malignant progenitor cells and for development as immunogens for polyvalent defined-antigen vaccines.

**Materials and Methods**

**Patient samples and cell preparation.** Heparinized blood samples were obtained from patients and normal donors enrolled on clinical research protocols at the Dana-Farber Cancer Institute (DFCI) approved by the DFCI Human Subjects Protection Committee. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll/Hypaque density gradient centrifugation, cryopreserved with 10% DMSO, and stored in vapor-phase liquid nitrogen until the time of analysis. Plasma was isolated following brief centrifugation of whole blood and cryopreserved at −80°C until analysis.

**Immunophenotypic analysis.** PBMCs collected from DLI-treated patients before and 3, 6, 9, and 12 mo following DLI were incubated for 30 min at 4°C with a panel of mouse monoclonal antibodies (CD20, CD19, CD5, CD3, CD6, CD56, CD14, and CD25). Cells were washed and incubated at 4°C with FITC goat anti-mouse conjugate (TAGO). Ten thousand cells falling within the lymphocyte gate were analyzed in each sample using automated flow cytometry (EPICS-V, Coulter Electronics). Percentages were applied to clinical leukocyte counts and differentials to calculate absolute cell numbers.

**Serologic screening of a CML cDNA phage library.** The construction and screening of a CML cDNA library has been previously described (4). Briefly, mRNA, extracted from PBMCs of three CML patients, was pooled to create a representative CML expression library in a λgt11 bacteriophage expression vector. Patient plasma (diluted 1:500) was incubated against nitrocellulose filters coated with isopropyl-D-thio-B-D-galactopyranoside–induced expressed products of recombinant phages comprising the library. Specific binding of antibody to recombinant proteins expressed on lytic plaques was detected by incubation with alkaline phosphatase-conjugated anti-human IgG antibody (1:2,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA).

**Figure 1.** GVL responses following DLI for treatment of CML are associated with B-cell immunity. A, significant CD20+ B-cell lymphocytosis was observed in DLI responders (n = 7; black circles; solid line, mean absolute B-cell number) at 6 and 9 mo post-DLI compared with pre-DLI levels (P = 0.03 and 0.04, respectively, two-sided exact Wilcoxon test) but not in DLI nonresponders (n = 5; gray squares; dashed line, mean absolute B-cell number) at any time point post-DLI. Absolute B-cell numbers were calculated by applying the percentage of CD20+ cells in the lymphocyte gate to clinical lymphocyte counts. B, representative comparisons of antibody reactivity in pre-DLI (Y axis) versus post-DLI (Y axis) plasma of a DLI nonresponder and responder tested against the protein microarray. Gray diamonds, raw fluorescence intensities of all protein spot-antibody complexes are plotted; black squares, protein spots with significantly higher reactivity post-DLI compared with pre-DLI.
Table 1. Clinical characteristics of CML patients treated with DLI

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>BMT conditioning regimen</th>
<th>Disease at time of DLI</th>
<th>DLI dose (CD4⁺ cells/kg)</th>
<th>BMT to cytotogenetic relapse (mo)</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>28/F</td>
<td>Cy/TBI</td>
<td>SP</td>
<td>3 × 10⁸</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td>47/M</td>
<td>Cy/TBI</td>
<td>SP</td>
<td>3 × 10⁷</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>40/F</td>
<td>Cy/TBI</td>
<td>SP</td>
<td>3 × 10⁷</td>
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</tr>
<tr>
<td>D</td>
<td>24/M</td>
<td>Cy/TBI</td>
<td>SP</td>
<td>1 × 10⁸</td>
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<tr>
<td>E</td>
<td>51/F</td>
<td>Cy/TBI</td>
<td>SP</td>
<td>3 × 10⁷</td>
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</tr>
<tr>
<td>F</td>
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<td>Cy/TBI</td>
<td>SP</td>
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</tr>
<tr>
<td>G</td>
<td>38/F</td>
<td>Cy/TBI</td>
<td>SP</td>
<td>3 × 10⁷</td>
<td>6</td>
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</tbody>
</table>

(Continued on the following page)

Immunoscreening using high-density protein microarrays. Commercial protein microarrays (Human ProtoArray version 3; Invitrogen) were probed with plasma samples and processed as previously described (5). All plasma samples from a single patient were screened with microarrays from the same printing lot. The microarrays contained ∼5,000 NH₂-terminal glutathione S-transferase fusion human proteins expressed in an insect cell line and spotted on nitrocellulose-coated glass slides. After blocking, patient plasma (diluted 1:50) was applied to the microarray surface for 90 min, and antibody-antigen interactions were detected with Alexa Fluor 647–conjugated anti-human IgG (H and L chain) antibody (1:2,000; Invitrogen). The microarrays were scanned at 5-μm resolution at 635 nm, 100% power, and 600 gain (GenePix 4000B scanner, Molecular Devices).

Lot-specific protein spot definitions provided by the microarray manufacturer were manually aligned to the image data. Fluorescence intensities were quantified using GenePix Pro 5.0 (Molecular Devices) with default settings. Signal change between pretreatment and posttreatment plasma was considered significant if change in both (a) signal magnitude ($Z_{\text{post}} - Z_{\text{pre}}$), and (b) ratio ($Z_{\text{post}}/Z_{\text{pre}}$), was greater than a cutoff, $n$ ($n = 5$ for significant antigens, called “candidate antigens”). The complete protein microarray screening data set is publicly available as a Gene Expression Omnibus data set (accession GSE11565).

Gene expression microarray data analysis. To compare gene expression of our collection of target antigens between normal and malignant CD34⁺ cells, raw data files (CEL) generated on Affymetrix HG-FOCUS microarrays (6), were collected and normalized using robust multiarray average (RMA) to create an expression matrix. A second expression matrix was generated from a series of CEL files on Affymetrix HG-U133A microarrays derived from multiple cell types: normal epithelial cells (7); monocytes, neutrophils, and acute myelogenous leukemia (AML) cells (8); CD34⁺ cells from normal donors (9–11); the K562 cell line; total PBMC (10); and total bone marrow (9, 12) from normal donors. These data sets were normalized using RMA followed by batch effect correction using the empirical Bayes method (13). To compare expression values, the two data matrices were globally scaled such that every array corresponding to a sample had the same median expression value. Gene identifiers of the panel of target antigens were mapped to Affymetrix probe set IDs using RESOURCERER (14). Median expression value was used for analysis if one target antigen mapped to multiple probe set IDs.

CEL files were also processed using Affymetrix MAS 5.0 program to generate detection calls for individual probe set IDs. Genes were defined as detectable for each cell type if detection calls for the corresponding probe set IDs were present in ≥40% of samples of that cell type. The relative detection difference between the panel of antigens and all the genes on the microarrays was analyzed using the Fisher’s exact test. Clustering analysis and expression visualization for individual genes within the scaled expression matrices were performed using the Multiexperiment Viewer software (15).

Primary cell lysates and Western blotting. Whole-cell lysate was generated from the K562 cell line or from patient samples by lysis in radioimmunoprecipitation assay buffer [1% NP40, 0.5% deoxycholate, 0.1% SDS, 125 mmol/L sodium chloride, and 50 mmol/L HEPES (pH 7.4)] in the presence of protease inhibitors. Lysates (20 μg per lane) were subjected to protein gel electrophoresis using 4% to 15% SDS-PAGE with Tris-glycine buffer and transferred onto nitrocellulose filters in Tris-glycine buffer with 20% methanol. Protein bands were visualized using antigen-specific antibodies against DUSP12 (1:4,000; Abcam), RAB38 (clone 11B-7, 1:200; Santa Cruz Biotechnology), and TBCE (1:500; ProteinTech Group). Antibody to β-actin (1:3,000; Sigma) was used as a control to ensure equal loading of lanes.

5 Provided by I. Bruns, unpublished data.
Immunoprecipitation assay. DNA sequences encoding a subset of the candidate antigens were either acquired in [Human ORFeome collection (16), DFCI] or cloned into Gateway expression-ready format (Invitrogen). Additional DNA sequences were purchased from Open Biosystems and cloned into pDONR221 (Invitrogen). DNA sequences were shuttled from the Gateway donor vector into a Gateway-converted mammalian expression vector containing a T7 promoter and a COOH-terminal GST tag (gift of Wagner Montor, Harvard Institute of Proteomics, Boston, MA). Candidate antigens were transcribed and expressed in vitro with rabbit reticulocyte lysate (TNT T7 Quick Coupled Transcription/Translation, Promega) using biotinylated lysine tRNA (Transcend tRNA, Promega), and expressed protein was immunoprecipitated with patient plasma as previously described (5).

Immunoprecipitated protein was detected on immunoblot using immunoperoxidase-streptavidin (1:20,000 dilution; MP Biomedicals). Blots were developed with SuperSignal West Femto chemiluminescence substrate (Pierce Biotechnology) and imaged on Kodak BioMax Light film. For the patient panels, low, moderate, or high reactivity was determined by visual inspection of bands on the developed blot. A constant quantity of streptavidin-labeled recombinant antigen was loaded onto each gel with immunoprecipitated recombinant antigen, and blots were developed to equivalent densities of the control antigen lane. Low reactivity was defined as reactivity at or below background levels; moderate reactivity as a clear band with equivalent or greater density than the control lane; high reactivity as a strong, black band with higher density than the control protein. Background

Table 1. Clinical characteristics of CML patients treated with DLI (Cont’d)

<table>
<thead>
<tr>
<th>Therapy before DLI</th>
<th>BMT to DLI (mo)</th>
<th>DLI to complete cytogenetic response (mo)</th>
<th>DLI to complete molecular response (mo)</th>
<th>GVHD after DLI</th>
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<tr>
<td>IFN</td>
<td>54</td>
<td>3</td>
<td>12</td>
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<td>IL-2, IFN</td>
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<tr>
<td>IL-2</td>
<td>42</td>
<td>2</td>
<td>7</td>
<td>No</td>
</tr>
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</table>

Abbreviations: Cy, cyclophosphamide (1,400 mg/m² × 2 d); TBI, fractionated total body irradiation 1,400 cGy in 7 fractions over 4 d; SP, stable-phase disease; NA, response not achieved; IL-2, interleukin-2.

Figure 2. Serologic screening of CML patients who responded to DLI, using two complementary immunoproteomic platforms, yields a large collection of candidate antigens. Cell surface-expressed proteins are shown in bold.

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GVL Targets Are Expressed on CML Progenitors
plasma reactivity was corrected for by examining plasma GST reactivity compared with reactivity against recombinant candidate antigens.

**Results**

**Identification of targets of GVL-associated humoral immunity.** Real-time immunophenotyping of seven CML patients who attained durable remissions following CD4+ DLI revealed a statistically significant peripheral B-cell lymphocytosis at 6 and 9 months following DLI ($P = 0.03$ and 0.04, respectively, two-sided exact Wilcoxon test), which was not observed among five similarly treated CML DLI nonresponders (Fig. 1A). No significant difference in absolute T cell, natural killer cell, or monocyte counts was observed between DLI responders and nonresponders after DLI (data not shown). As shown in Table 1, these seven DLI-responsive patients (A–G) comprised a homogenous clinical group: all patients relapsed 24 to 52 months following myeloablative allogeneic HSCT, received CD8-depleted donor lymphocytes for the treatment of relapsed stable-phase CML (17), and promptly developed cytogenetic and molecular responses (median, 3.5 and 8 months post-DLI, respectively). None developed clinically significant GVHD.

To identify the antigen targets of DLI-associated B-cell responses, we probed two different immunoproteomic platforms with plasma from the DLI-responsive patients collected at 1 year post-DLI. CML expression library screening was performed using plasma samples from all seven patients, whereas the protein microarray experiments were restricted to patients A, B, and C. For both platforms, target antigens were defined as proteins eliciting new or increased antibody reactivity in post-DLI compared with pre-DLI plasma.

A total of 62 DLI-associated antigens were identified using the two screening methods. Expression library screening identified 22 distinct target antigens (Fig. 2; Supplementary Table S1A), with one to eight antigens identified per patient. Protein microarray screening using DLI responder plasma identified an additional 40 candidate antigens (Fig. 2; Supplementary Table S1B). One protein identified by protein microarray screening (MMAB) and three proteins identified by library screening (DEFA1, PTK2, and RBPJK) were recognized by two or more patients. No candidate antigens were identified by protein microarrays when screened with three distinct DLI nonresponder plasma samples (representative example shown in Fig. 1B).

Minimal overlap was observed between antigens identified by expression library and protein microarray screening (Fig. 2). Only two of the 22 antigens identified by expression library screening, GPKOW and EXOSC5, appeared in equivalent form within the content of the protein microarray. Plasma from a single individual (patient A) identified these antigens by both screening methods, showing consistency between these complementary platforms. Most identified antigens were proteins of known function (Supplementary Table S1), including nucleic acid binding, signal transduction, cellular proliferation and differentiation, cell cycle regulation, host defense and immune function, and cytoskeletal dynamics. Nearly half of the known antigens identified by expression library screening were involved in DNA or RNA binding, whereas antigens identified by protein microarray screening had more diverse functions. All antigens identified by expression library screening were intracellular. Protein microarray screening uncovered five cell surface antigens (RET, ACVR2B, TNFRSF19, TGFB2, and TYRO3) as well as intracellular antigens.

**Gene expression of DLI-associated antigens in hematopoietic and nonhematopoietic cells.** To assess whether the identified antigens represented genes with enriched expression in CML progenitor cells, we examined their mRNA transcript expression in hematopoietic and nonhematopoietic cells. We generated a single expression matrix composed of expression data from normal myeloid lineage cells (monocytes

![Image](327x241 to 528x499)

Figure 3. Detection of gene expression of candidate antigens in cell populations. A, using an expression matrix of data sets generated on the HG-U133A microarray, the percent of candidate antigens with detectable transcript expression (black columns) in cells of hematopoietic and nonhematopoietic lineage was compared with the percentage of genes sampled by the gene microarray with detectable gene expression for each cell type (white columns). Significance was determined using the Fisher’s exact test. NL, normal; BM, bone marrow; PMNs, polymorphonuclear leukocytes. B, percentage of microarray genes detected as transcribed in normal and CML CD34+ cells. White columns, overall microarray genes; gray columns, candidate antigens identified by cDNA expression library screening; black columns, candidate antigens identified by protein microarray screening. Gene microarray data were generated from CD34+ cells isolated from 12 CML patients and 8 normal volunteers (6).
and malignant myeloid lineage cells (AML cells and K562 cells, a BCR-ABL-positive CML cell line), normal hematopoietic tissue (PBMC and bone marrow), and normal epithelial cells.

The percentage of genes detected on a given array varies significantly depending on the type of array, the tissue or cell types assayed, and the RNA quality.6 We therefore compared detection of antigens of interest against the overall detection of all genes tested by the array for each cell type. To validate this method, we applied the expression matrix to two known leukemia-associated antigens: WT-1 and proteinase 3 (18). Predictably, these antigens showed elevated expression in K562 cells and progenitor cells and depressed expression in mature myeloid cell and nonhematopoietic cell populations (data not shown) in this expression matrix.

Next, we evaluated transcript expression of those 43 antigens from our collection of 62 DLI-associated antigens (69%) for which probe sets were present on HG-U133A arrays. Depending on the cell type examined, transcripts from 27% to 54% of the 22,215 total genes represented on the HG-U133A arrays were detected (Fig. 3A). A higher than expected detection of transcripts of our DLI-associated antigens was observed in hematopoietic tissue only, particularly in early myeloid lineage cells. Compared with the array-wide rate of detection of gene transcription, 63% of our panel of antigens was expressed in normal CD34+ cells (P = 0.07), and 65% (P = 0.02) in K562 cells. In more differentiated myeloid lineage cells (monocytes and neutrophils) and in nonhematopoietic tissue, detection of candidate antigen gene expression was not significantly elevated (Fig. 3A).

Candidate antigens are commonly detected in CD34+ cells. To confirm enriched expression of DLI-associated antigens in CML progenitor cells, we compared gene expression of these antigens between primary normal and malignant CD34+ cells. The data set for this analysis was generated by applying RNA from CD34+ cells isolated from patients with untreated stable-phase CML (n = 12) and from normal donors (n = 8) to Affymetrix HG-FOCUS arrays (6). Gene expression analysis in CD34+ cells was restricted to the 45% of the expression library and 69% of the protein microarray–identified antigens for which probe sets were present on the HG-FOCUS arrays (39 antigens total).

Consistent with the HG-U133A expression matrix studies, detection of candidate antigens was highly enriched in both normal and malignant CD34+ cells. Seventy percent (7 of 10) of antigens identified by expression library screening were detected in normal CD34+ cells, and 80% (8 of 10) were detected in CML CD34+ cells (Fig. 3B). These levels of detection were significantly higher (P = 0.04 and 0.02, respectively) than the array-wide detection rate of 48% to 54% of the 8,746 genes represented on the array in normal or malignant CD34+ cells. Similarly, protein microarray–identified antigens were detected in greater than expected numbers on normal CD34+ cells (18 of 29, 62%; P = 0.04) and on CML CD34+ cells (19 of 29, 66%; P = 0.08) compared with array-wide detection.

RAB38, TBE, and DUSP12 are preferentially expressed in CML CD34+ cells. Because antigens that are both naturally immunogenic and differentially overexpressed in malignant progenitor cells may be the most promising immunotherapeutic targets, we examined the relative gene expression of the candidate antigens on the HG-FOCUS and HG-U133A Affymetrix microarrays. This analysis revealed a subset with differential expression between normal and malignant progenitor cells (Fig. 4; Supplementary Table S2). DUSP12, RAB38, RP56K1, TBE, and VPS4B had significantly enriched expression in CML compared with normal CD34+ cells (P ≤ 0.002, Student’s t test). Examination of myeloid progenitor cell subsets (19) in CML and normal donors revealed higher expression of these five proteins in the majority of progenitor cell subsets in CML compared with normal CD34+ cells (Supplementary Table S3). Expression of all five was low in normal total bone marrow mononuclear cells as well as in normal differentiated hematopoietic cell types (monocytes, neutrophils, and PBMC). All five antigens had high relative

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6 F. Liu, manuscript in preparation.
expression in the K562 CML cell line. In contrast, expression of these antigens was low in mature, differentiated hematopoietic cell types (monocytes, neutrophils, and PBMC) compared with malignant progenitors.

Of these five antigens, only DUSP12, RAB38, and TBCE were validated as immunogenic by plasma immunoprecipitation assay (Fig. 5A). Subsequent studies thus focused on characterization of these three antigens. To confirm the results of the gene expression microarray analysis, transcript levels were measured directly by quantitative reverse transcription-PCR using mRNA extracted from normal and CML CD34+ cells, and all three antigens were expressed at ~2-fold higher median levels in CML CD34+ cells compared with normal CD34+ cells (Supplementary Fig. S1). At the protein level, we observed greater expression of all three antigens in CD34+ progenitor compared with CD34− nonprogenitor cell populations on Western blotting of marrow lysates generated from normal volunteers (n = 4) and CML patients (n = 4). Representative examples are shown in Fig. 5B. Differential expression between normal and CML progenitor cells was most striking for DUSP12. In addition, expression of DUSP12 and TBCE was also seen in primary AML cells and the K562 CML cell line.

**DUSP12, RAB38, and TBCE elicit treatment-associated immunity in patients with CML.** To further examine the relationship between remission in patient C and the development of antibody reactivity against DUSP12, RAB38, and TBCE, we tested serial pre-DLI versus post-DLI plasma samples from patient C for their ability to immunoprecipitate recombinant antigens translated in vitro. As shown in Fig. 5A, DUSP12, RAB38, and TBCE each transiently elicited greater binding by post-DLI plasma samples compared with the pre-DLI sample. Positive reactivity against

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**Figure 5.** DUSP12, RAB38, and TBCE have enriched expression in CML CD34+ cells and elicit antibody responses in patients developing treatment responses against CML. A, post-DLI plasma of patient C has greater reactivity than either pre-HSCT or pre-DLI against DUSP12, RAB38, and TBCE, shown by immunoprecipitation and Western blotting. Antibody reactivity was temporally correlated with response to therapy (PCR, molecular detection of BCR-ABL transcript; Cyto, detection of the Philadelphia chromosome). The EBV-derived protein EBNA1 was used as a positive control antigen. B, increased protein expression of DUSP12, RAB38, and TBCE was seen in CML CD34+ cells and other malignant myeloid cells compared with normal CD34− cells and CML CD34− cells. Increased protein expression of DUSP12 was also seen in CML CD34+ cells compared with normal CD34− cells, although protein expression of RAB38 and TBCE was equivalent in both normal and malignant CD34+ cells. C, antibody reactivity against recombinant GST-tagged DUSP12, RAB38, and TBCE is observed in other patients with effective CML immunity but not in normal donors and only rarely in allotransplanted patients without CML, as shown by immunoprecipitation of individual recombinant proteins with patient plasma followed by Western blotting. Dark gray shading, strong plasma binding of antigen; light gray shading, moderate plasma binding of antigen; no shading, plasma binding at or below background levels. Data are normalized to plasma GST binding.
DUSP12 was detected at 1 month and persisted until 3 months following DLI. Antibody responses against RAB38 and TBCE arose at 3 months post-DLI, corresponding to when the patient developed cytogenetic remission. These antibody responses persisted until 9 to 12 months after DLI when the patient developed molecular remission. Consistent with the elimination of an immunogen, responses against DUSP12, RAB38, and TBCE waned after 12 months post-DLI and were undetectable 3 to 4 years after DLI. In contrast, the positive control EBV-derived EBNA1 protein was immunoprecipitated at relatively equivalent amounts by all plasma samples tested.

To confirm the association between immunity against candidate antigens and effective CML elimination, we used the same immunoprecipitation-based assay to perform an expanded serologic analysis of CML patients undergoing various therapies. As shown in Fig. 5C, none of seven normal donors (group 5) developed antibody reactivity against DUSP12, RAB38, or TBCE above background, whereas all seven plasma samples from CML patients with GVL responses (group 1) showed a high frequency of binding to the three candidate antigens (five, four, and six of seven for DUSP12, RAB38, and TBCE, respectively). These seven patients (three previously unexamined patients as well as patients B, D, F, and G) all achieved durable molecular remissions following DLI and did not develop significant GVHD. In contrast, antibody reactivity against candidate antigens was present, but at lower frequencies, in seven imatinib-responsive CML patients (group 2; four, three, and three of seven for DUSP12, RAB38, and TBCE, respectively) and in seven CML patients who achieved complete remission after HSCT GVHD (group 3; one, one, and four of seven for DUSP12, RAB38, and TBCE, respectively). Antibody responses against DUSP12, RAB38, and TBCE thus seem specific to effective CML immunity, as they were not frequently seen with either non-immune-mediated tumor destruction (imatinib, group 2) or nonspecific immunity (GVHD, group 3). To assess the disease specificity of antibody responses against our candidate antigens, we identified six additional patients with effective tumor immunity against a nonmyeloid malignancy (group 4). These patients achieved complete remission of chronic lymphocytic leukemia (CLL) following HSCT and DLI without GVHD. None of the six patients in group 4 was reactive to DUSP12 or RAB38 but showed relatively high reactivity (four of seven) against TBCE. Taken together, these studies suggest that DUSP12, RAB38, and TBCE are broadly immunogenic in patients with effective tumor immunity.

Discussion

Tumor-initiating cells have been identified for several hematologic malignancies, including CML (20). To date, approved pharmacologic therapies for CML do not effectively eliminate the malignant progenitor cell (21, 22). A persistent malignant progenitor population ensures a continued risk for developing disease resistance and relapse, as evidenced by a continuous relapse rate of 2% to 3% per year in patients on imatinib therapy (23). Given the susceptibility of CML to immune-mediated destruction and the exquisite specificity of the adaptive immune system, immunotherapy directed at CML progenitor cell targets has the potential to effectively eliminate these cells without concurrent toxicity. Critical to this effort is the identification of antigens specific to the malignant progenitor cell population.

We focused on natural immune responses developing within a clinically homogenous group of patients who experienced clear curative responses (without GVHD) following DLI to reveal targets expressed on progenitor cells. We observed that patients responsive to DLI consistently developed B-cell lymphocytosis and high-titer plasma antibodies that were not present before therapy or in patients unresponsive to DLI. The timing of these B-cell responses closely correlates with clinical and cytogenetic responses against CML and potentially represents developing immunity against lysed tumor cells or their extruded contents. We therefore used established and recently available immunoproteomic platforms to identify 62 novel targets of B-cell immunity following DLI. Some patient samples were concurrently screened by both cDNA expression library and by protein microarray, and each platform yielded different collections of target antigens. In particular, bacteriophage expression library screening exclusively identified intracellular antigens, similar to previously published studies using this screening strategy (24, 25). In contrast, protein microarray screening yielded antigens with both intracellular and surface expression. The two platforms thus represent complementary tools for detecting serologic responses. Nonetheless, the union of the data sets revealed that, irrespective of platform, a feature common to the entire collection was detectable expression on hematopoietic cells generally and myeloid progenitor cells specifically. By using the strategy of first discovering immunogenic targets by serologic screening, and then focusing on those with high transcript expression in CML CD34+ cells, we successfully identified a subset of antigens that commonly elicit immune responses among other CML patients with GVL responses.

This antigen subset included RAB38, TBCE, and DUSP12. RAB38, a small G protein involved in endoplasmic reticulum–related vesicle transport, generates specific antibody (26) and T-cell (27) responses in melanoma patients and is highly expressed in melanoma tissue and absent in normal tissue (28). TBCE is a chaperone for tubulin heterodimerization and microtubule polymerization; individuals with mutations in this gene show defective neutrophil migration and phagocytosis (29). DUSP12, the human orthologue of the yeast YHV1 protein tyrosine phosphatase, may be involved in cell growth regulation (30). Of the three candidate antigens, antibody responses against DUSP12 were the most clearly associated with CML; antibody responses against this antigen were seen in more than half the CML patients with GVL studied. Antibody responses against RAB38 also seemed specific to CML. Interestingly, patients with effective immunity against another hematologic malignancy, CLL, had a similar frequency of antibody responses against TBCE compared with CML patients with effective immunity. TBCE may
represent a common GVL-associated immunogen between these two disorders.

RAB38, TBCE, and DUSP12 and many other target antigens identified by our serologic screens are intracellular proteins. Antibody responses against these antigens presumably arise as a result of cell lysis, providing access of intracellular contents to the immune system. Recently, several transplant and vaccination studies have shown that development of antigen-specific B-cell responses strongly correlated with clinical response (31–33). Because immunity can develop against extruded cellular contents following a cytotoxic response, antibody responses against particular antigens can be reliable biomarkers of effective tumor destruction. This notion is supported by the results of our immunoprecipitation studies (Fig. 5C) because CML patients who developed remissions after receiving both immune and pharmacologic therapy commonly developed antibody responses to DUSP12, RAB38, and TBCE, with the greatest frequency and intensity in patients with GVL responses. Alternatively, several studies have supported an active role for antibodies against intracellular antigens in enhancing cellular immunity by facilitating cross-presentation of antigen to T cells (34–37). In the setting of GVL-induced clinically evident immunity, it is likely that coordinated humoral and cellular immunity against leukemia-associated antigens occurs. Therefore, our future studies will explore whether RAB38, DUSP12, and TBCE can elicit T-cell responses that are lytic to CML CD34+ cells.

Similar to the previously identified DLI-associated antigens CML66 and CML28 (3), most newly identified antigens showed no significant difference in relative gene expression between normal and malignant CD34+ cells. BCR-ABL kinase activity seems to be crucial for the enhancement of immunogenicity of CML cells (38, 39), and the BCR-ABL–positive K562 cell line possesses both standard and immunoproteasomal processing capability (40). Because we identify antigen-specific immune responses in patients who have developed GVL but not GVHD, we speculate that CML CD34+ cells potentially process and present antigen differently than normal CD34+ cells, which in turn may enable T cells to discriminate between normal and malignant cells. In support of this concept, cytolytic T lymphocytes specific for the CML-associated antigen proteinase 3 are cytotoxic against CML CD34+ cells but not normal bone marrow–derived CD34+ cells despite high expression in both malignant and normal progenitor cell populations (41). In the presence of high-titer antigen-specific antibody responses developing following DLI, it is likely that CD4+ T-cell responses against CML antigens are also present. However, we have not addressed this question to date.

Our discovery of a large panel of immunogenic antigens present on CML CD34+ cells opens the door for immune monitoring of therapies designed to immunologically target CML progenitor cells, such as stem cell transplantation and whole tumor cell–based vaccines (42). Because the precise immunogens targeted by cell-based treatment strategies are unknown, monitoring immune responses with a large collection of immunogenic progenitor cell–associated antigens would be informative. Finally, with further testing to determine the cytolytic potential and helper function of T cells directed against our identified antigens, our panel of targets may provide a starting point for studies to explore strategies to stimulate polyvalent antigen-specific immunity against malignant CML progenitor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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