# Antigen targets of remission-inducing immune therapy are expressed on CML progenitor cells

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## Abstract

The curative effect of existing graft-versus-leukemia (GvL)-based therapies such as donor lymphocyte infusion (DLI) for chronic myeloid leukemia (CML) may result from immunologic targeting of self-renewing CML progenitor cells. Patients who achieved durable remission following DLI developed a significant B cell lymphocytosis after treatment, which was not observed in patients unresponsive to DLI. To identify targets of this B cell response, we probed two complementary immunoproteomic platforms, a bacteriophage expression library and a high-density protein microarray, using plasma immunoglobulin from seven CML patients with clinically apparent GvL and without graft-versus-host disease after DLI. In total, 62 antigens elicited greater reactivity from post-DLI versus pre-DLI plasma. More than 70% of the antigens were detectable in CML CD34+ cells in an analysis of HG-FOCUS and HG-U133A Affymetrix microarrays, suggesting that expression in malignant progenitor cells is a feature common to antibody targets of DLI. Higher transcript and protein expression in CML compared to normal CD34+ cells was confirmed for three target antigens (RAB38, TBCE, and DUSP12). Together, they consistently elicited antibody responses in an additional 18 of 21 CML patients with clinical responses to therapy, but not in normal donors and only rarely in patients without CML. Immunologic targets of curative DLI responses thus comprise multiple CML progenitor cell-expressed antigens that may represent potential immunogens for vaccination and/or monitoring of immunotherapeutic strategies designed to eliminate myeloid leukemia stem cells.

## Keywords

Donor lymphocyte infusion; chronic myeloid leukemia; hematopoietic stem cell; protein microarray; graft-versus-leukemia

## INTRODUCTION

The elimination of minimal residual disease remains a critical issue in the treatment of patients with chronic myeloid 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 anti-tumor effects of GvL responses. In this therapy, donor mononuclear cells are infused after allogeneic HSCT in the absence of further chemo- or radiotherapy. Durable disease remission is achieved in 75–80% of patients with relapsed CML treated with DLI.[2] Since 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 anti-tumor 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 datasets of normal and malignant hematopoietic tissue confirmed that more than 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 to their normal equivalents. As a collection, these antigens potentially represent highly informative reagents for immunological 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

PBMC collected from DLI-treated patients before and 3, 6, 9, and 12 months following DLI were incubated for 30 minutes at 4C with a panel of mouse monoclonal antibodies (CD20, CD19, CD5, CD3, CD6, CD56, CD14, and CD25). Cells were washed and incubated at 4C with fluorescein-isothiocyanate (FITC) goat anti-mouse conjugate (TAGO, Burlingame, CA). Ten thousand cells falling within the lymphocyte gate were analyzed in each sample using automated flow cytometry (EPICS-V, Coulter Electronics, Hialeah, FL). 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 PBMC 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 IPTG-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 (1:2000 dilution; Jackson ImmunoResearch, West Grove, PA), followed by incubation with detection reagent (BCIP/NBT; Promega, Madison, WI). cDNA inserts from positive clones were excised from phagemids and sequenced with T3 and T7 primers. Sequence comparisons were performed by BLAST search against GenBank databases.

## Immunoscreening using high-density protein microarrays

Commercial protein microarrays (Human ProtoArray, v3; Invitrogen, Carlsbad, CA) 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 approximately 5000 N-terminus GST-fusion human proteins expressed in an insect cell line and spotted on nitrocellulose-coated glass slides. After blocking, patient plasma (diluted 1:150) was applied to the microarray surface for 90 minutes, and antibody-antigen interactions detected with AlexaFluor 647-conjugated anti-human IgG (H and L chain) antibody (1:2000; Invitrogen, Carlsbad, CA). The microarrays were scanned at 5μm resolution at 635 nm, 100% power and 600 gain (GenePix 4000B scanner; Molecular Devices, Sunnyvale, CA).

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, Sunnyvale, CA) with default settings. Signal change between pre- and post-treatment plasma was considered significant if change in both (1) signal magnitude ($Z_{delta}$), defined as $Z_{post}$–max(0, $Z_{pre}$), and (2) ratio ($Z_{mult}$), defined as $Z_{post}$/max(1,$Z_{pre}$), was greater than a cutoff, $n$ ($n$=5 for significant antigens, called "candidate antigens"). The complete protein microarray screening dataset is publicly available as a Gene Expression Omnibus dataset (GEO 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 AML cells;[8] CD34+ cells from normal donors;[9–11] the K562 cell line (provided by I. Bruns, unpublished data); total PBMC[10] and total bone marrow[9, 12] from normal donors.

These datasets 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 probeset IDs (PsIDs) using Resourcerer.[14] Median expression value was used for analysis if one target antigen mapped to multiple PsIDs.

CEL files were also processed using Affymetrix MAS 5.0 program to generate detection calls for individual PsIDs. Genes were defined as detectable for each cell type if detection calls for the corresponding PsIDs 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 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 RIPA buffer (1% NP-40, 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 4–15% 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 (1:4000; Abcam, Cambridge, MA), RAB38 (clone 11B-7, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and TBCE (1:500; ProteinTech Group, Chicago, IL). Antibody to β-actin (1:3000; Sigma, St. Louis, MO) was used as a control to ensure equal loading of lanes.

### 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, Carlsbad, CA). Additional DNA sequences were purchased from Open Biosystems and cloned into pDONR221 (Invitrogen, Carlsbad, CA). DNA sequences were shuttled from the Gateway donor vector into a Gateway-converted mammalian expression vector containing a T7 promoter and a C-terminus glutathione-S-transferase (GST) tag (gift of Wagner Montor, Harvard Institute of Proteomics). Candidate antigens were transcribed and expressed *in vitro* with rabbit reticulocyte lysate (TNT T7 Quick Coupled Transcription/Translation; Promega, Madison, WI) using biotinylated lysine transfer RNA (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, Solon, OH). Blots were developed with SuperSignal West Femto chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) 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 plasma reactivity was corrected for by examining plasma GST reactivity compared to reactivity against recombinant candidate antigens.

# RESULTS

## Identification of targets of GvL-associated humoral immunity

Real-time immunophenotyping of 7 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 5 similarly treated CML DLI non-responders (Figure 1A). No significant difference in absolute T cell, NK 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 one 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 to 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 (Figure 2; Supplemental Table 1A), with one to eight antigens identified per patient. Protein microarray screening using DLI responder plasma identified an additional 40 candidate antigens (Figure 2; Supplemental Table 1B). 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 non-responder plasma samples (representative example shown in Figure 1B).

Minimal overlap was observed between antigens identified by expression library and protein microarray screening (Figure 2). Only two of the twenty-two 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, demonstrating consistency between these complementary platforms. Most identified antigens were proteins of known function (Supplemental Table 1), 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, while antigens identified by protein microarray screening had more diverse functions. All antigens identified by expression library screening were intracellular. Protein microarray screening uncovered 5 cell surface antigens (RET, ACVR2B, TNFRSF19, TGFBR2, and TYRO3) as well as intracellular antigens.

## Gene expression of DLI-associated antigens in hematopoietic and non-hematopoietic 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 non-hematopoietic cells. We generated a single expression matrix composed of expression data from normal myeloid lineage cells (monocytes, neutrophils), 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 (F. Liu, personal communication, manuscript in preparation). 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 non-hematopoietic 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 probesets were present on HG-U133A arrays. Depending on the cell type examined, transcripts from 27–54% of the 22215 total genes represented on the HG-U133A arrays were detected (Figure 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 to 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 non-hematopoietic tissue, detection of candidate antigen gene expression was not significantly elevated (Figure 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 dataset 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 probesets 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. 70% (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 (Figure 3B). These levels of detection were significantly higher ($p=0.04$ and 0.02, respectively) than the array-wide detection rate of 48–54% of the 8746 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 (62% (18 of 29), $p = 0.04$) and on CML CD34+ cells (66% (19 of 29), $p = 0.08$), compared to array-wide detection.

## RAB38, TBCE, and DUSP12 are preferentially expressed in CML CD34+ cells

Since 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 (Figure 4, Supplemental Table 2). DUSP12, RAB38, RPS6KC1, TBCE, and VPS4B had significantly enriched expression in CML compared to normal CD34+ cells ($p=<0.002$, Student 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 to normal (Supplemental Table 3). Expression of all five was low in normal total bone marrow mononuclear cells, as well as in normal differentiated hematopoietic cell types (monocytes, neutrophils, PBMC). All five antigens had high relative 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 to malignant progenitors.

Of these 5 antigens, only DUSP12, RAB38, and TBCE were validated as immunogenic by plasma immunoprecipitation assay (Figure 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 RT-PCR using mRNA extracted from normal and CML CD34+ cells, and all three antigens were expressed at approximately two-fold higher median levels in CML CD34+ cells compared to normal CD34+ cells (Supplemental Figure 1). At the protein level, we observed greater expression of all three antigens in CD34+ progenitor compared to CD34− non-progenitor cell populations on Western blotting of marrow lysates generated from normal volunteers (n=4) and CML patients (n=4). Representative examples are shown in Figure 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- versus post-DLI plasma samples from Patient C for their ability to immunoprecipitate recombinant antigens translated *in vitro*. As shown in Figure 5A, DUSP12, RAB38, and TBCE each transiently elicited greater binding by post-DLI plasma samples compared to the pre-DLI sample. Positive reactivity against DUSP12 was detected at one month and persisted until 3 months following DLI. Antibody responses against RAB38 and TBCE arose at three months post-DLI, corresponding to when the patient developed cytogenetic remission. These antibodies responses persisted until 9–12 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 3–4 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 Figure 5C, none of 7 normal donors (Group 5) developed antibody reactivity against DUSP12, RAB38 or TBCE above background, whereas all 7 plasma samples from CML patients with GvL responses (Group 1) showed a high frequency of binding to the three candidate antigens (5, 4 and 6 of 7 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 7 imatinib-responsive CML patients (Group 2; 4, 3 and 3 of 7 for DUSP12, RAB38 and TBCE, respectively) and in 7 CML patients who achieved complete remission after HSCT GvHD (Group 3; 1, 1 and 4 of 7 for DUSP12, RAB38 and TBCE, respectively). Antibody responses against DUSP12, RAB38, and TBCE thus appear specific to effective CML immunity, as they were not frequently seen with either non-immune-mediated tumor destruction (imatinib, Group 2) or non-specific 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 non-myeloid malignancy (Group 4). These patients achieved complete remission of chronic lymphocytic leukemia (CLL) following HSCT and DLI without GvHD. None of the 6 patients in Group 4 were reactive to DUSP12 or RAB38, but showed

relatively high reactivity (4 of 7) 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 2–3% 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 prior to 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 datasets 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 over half the CML patients with GvL studied. Antibody responses against RAB38 also appeared specific to CML. Interestingly, patients with effective immunity against another hematologic malignancy, chronic lymphocytic leukemia, had a similar frequency of antibody responses against TBCE compared to 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 (Figure 5C), since 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 appears 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] Since we are identifying 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] Since 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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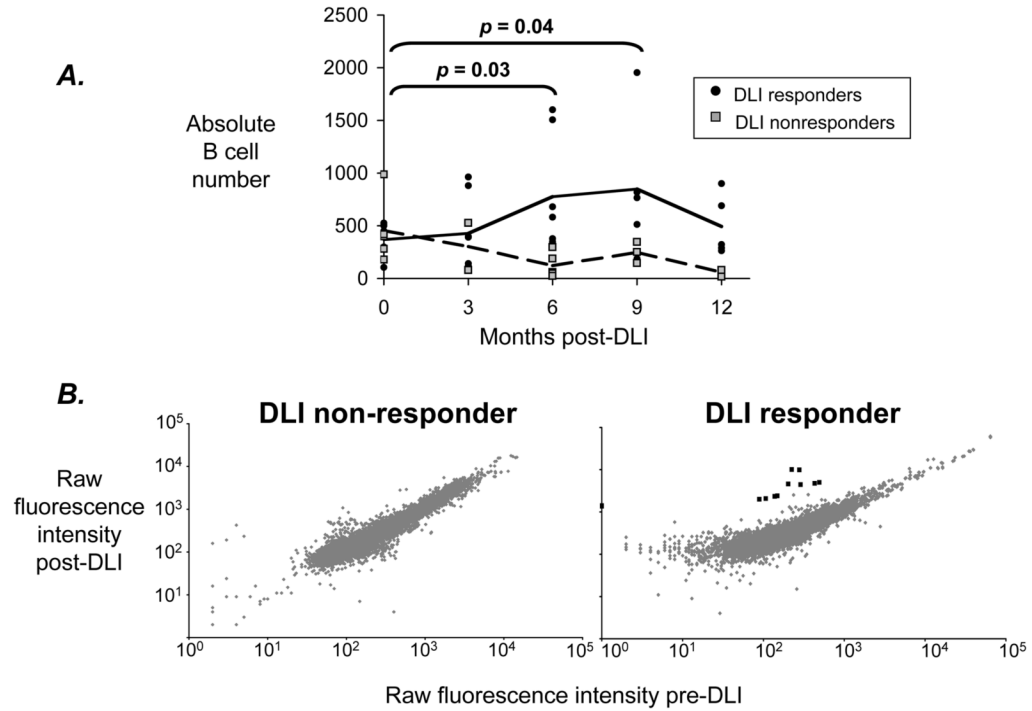
**Figure 1. GvL responses following DLI for treatment of CML are associated with B cell immunity**
**A.** Significant CD20+ B cell lymphocytosis was observed in DLI responders (n = 7; black circles; solid line=mean absolute B cell number) at 6 and 9 months post-DLI compared to pre-DLI levels (p=0.03; p=0.04, respectively; two-sided exact Wilcoxon test), but not in DLI nonresponders (n = 5; grey squares; dashed line=mean absolute B cell number) at any timepoint 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- (*x* axis) versus post-DLI (*y* axis) plasma of a DLI nonresponder and responder tested against the protein microarray. Raw fluorescence intensities of all protein spot-antibody complexes are plotted (grey diamonds). Protein spots with significantly higher reactivity post-DLI compared to pre-DLI are shown as black squares.
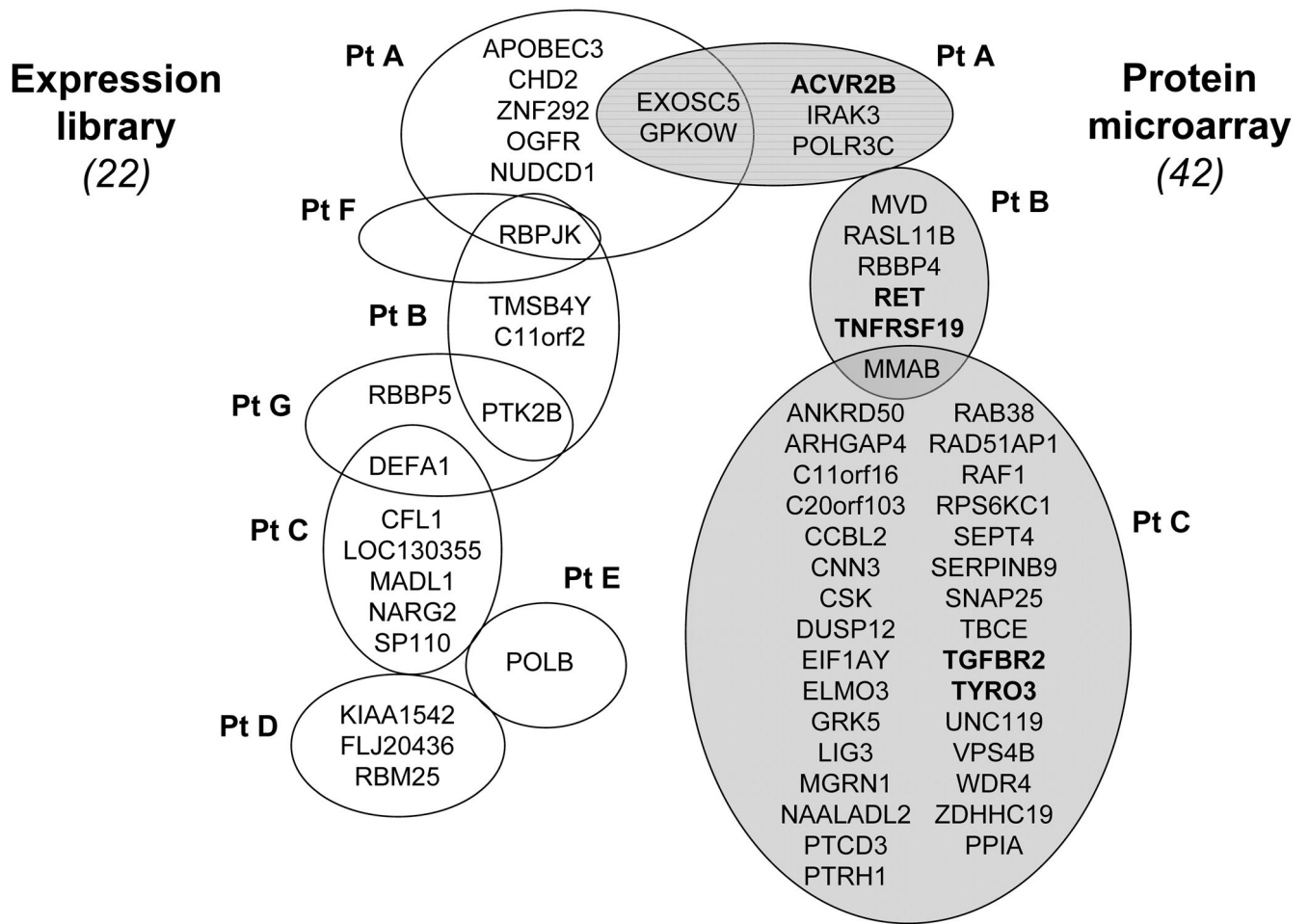
**Figure 2. Serologic screening of CML patients who responded to DLI, using two complementary immunoproteomic platforms, yields a large collection of candidate antigens**
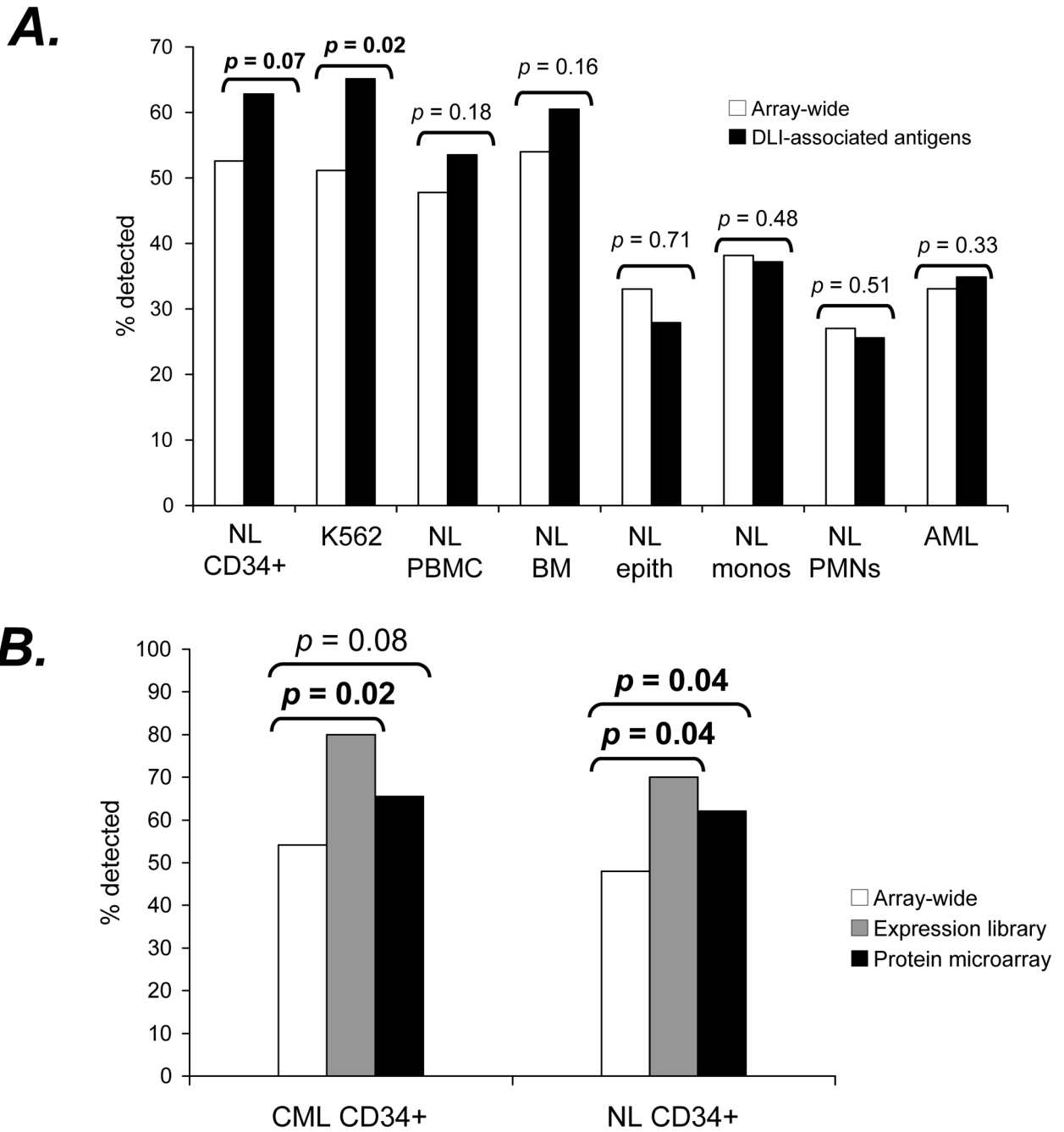Cell surface-expressed proteins are shown in bold.

**A.**



**B.**



**Figure 3. Detection of gene expression of candidate antigens in cell populations**
**A.** Using an expression matrix of datasets generated on the HG-U133A microarray, the percent of candidate antigens with detectable transcript expression (black bars) in cells of hematopoietic and non-hematopoietic lineage was compared with the percentage of genes sampled by the gene microarray with detectable gene expression for each cell type (white bars). Significance was determined using the Fisher Exact test. **B**. Percentage of microarray genes detected as transcribed in normal (NL) and CML CD34+ cells. Shown are overall microarray genes (white bars), candidate antigens identified by cDNA expression library screening (gray bars), and candidate antigens identified by protein microarray screening (black bars). Gene

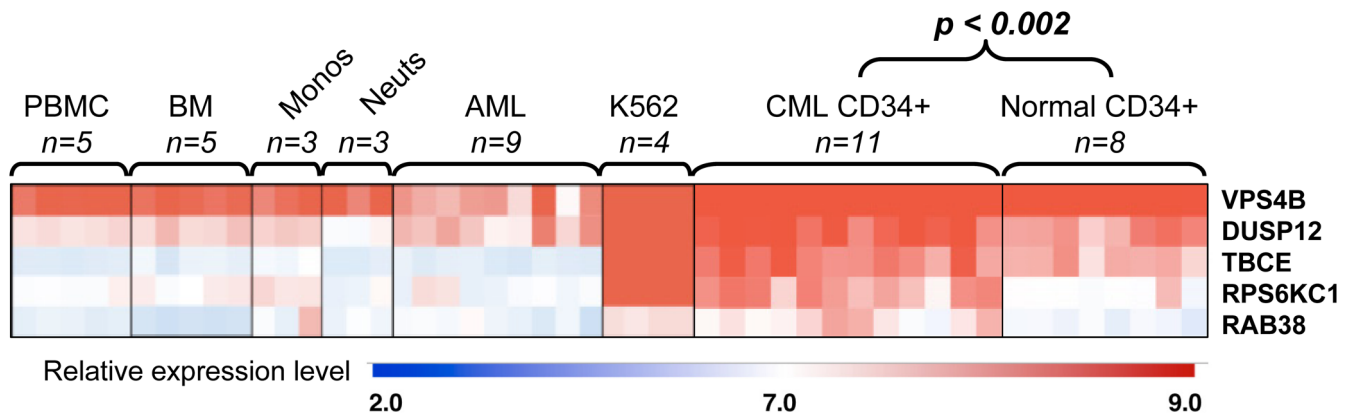microarray data was generated from CD34+ cells isolated from 12 CML patients and 8 normal volunteers [6].

**Figure 4. VPS4B, DUSP12, RAB38, RPS6KC1 and TBCE are overexpressed in CML progenitor cells**

Relative gene expression was detected by gene expression microarrays, among peripheral blood mononuclear cells (PBMC, n=5), bone marrow (BM, n=5), normal monocytes (Monos, n=3), granulocytes (Neuts, n=3), acute myeloid leukemia (AML, n=9), a CML cell line (K562, n =4), CML CD34+ cells (n=12), and normal CD34+ cells (n = 8). Significance was determined by Student T test.
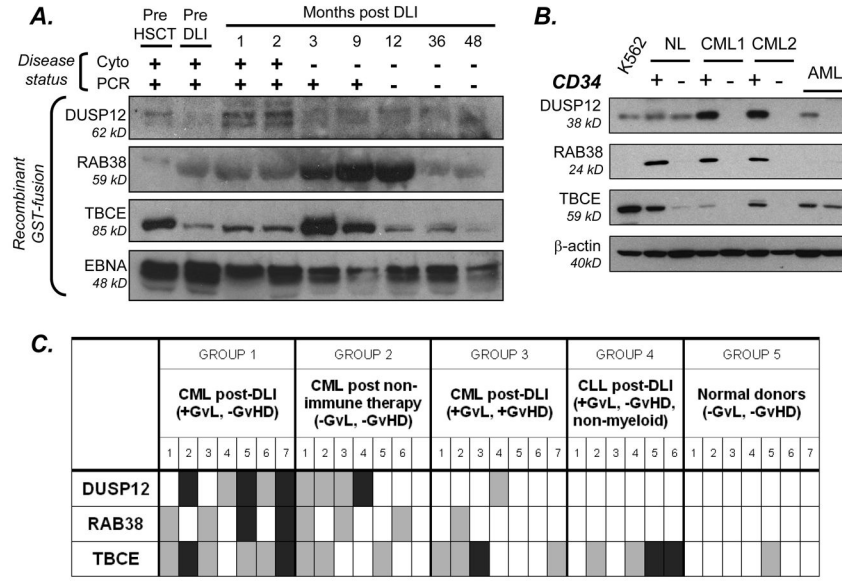
**Figure 5. DUSP12, RAB38, and TBCE have enriched expression in CML CD34+ cells and elicit antibody responses in patients developing treatment responses against CML**

**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 Philadephia 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 to normal CD34− cells and CML CD34− cells. Increased protein expression of DUSP12 was also seen in CML CD34+ cells compared to 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 demonstrated by immunoprecipitation of individual recombinant proteins with patient plasma followed by Western blotting. Dark grey shading indicates strong plasma binding of antigen, light grey shading indicates moderate plasma binding of antigen, and no shading indicates plasma binding at or below background levels. Data are normalized to plasma GST binding.

**Table 1**

Clinical characteristics of CML patients treated with donor lymphocyte infusion

| Pt | Age/sex | BMT conditioning regimen | Disease at time of DLI | DLI dose (CD4+ cells/ kg) | BMT to cytogenetic relapse (months) | Therapy before DLI | BMT to DLI (months) | DLI to complete cytogenetic response (months) | DLI to complete molecular response (months) | GVHD after DLI |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 28/F | Cy/TBI | SP | $3 \times 10^8$ | 11 | IFN | 54 | 3 | 12 | No |
| B | 47/M | Cy/TBI | SP | $3 \times 10^7$ | 12 | IL-2, IFN | 37 | 4 | 8 | No |
| C | 40/F | Cy/TBI | SP | $3 \times 10^7$ | 24 | none | 26 | 3.5 | 3.5 | Min |
| D | 24/M | Cy/TBI | SP | $1 \times 10^8$ | 13 | none | 27 | 4 | 5 | No |
| E | 51/F | Cy/TBI | SP | $3 \times 10^7$ | 13 | IL-2, IFN | 32 | 3 | 12 | No |
| F | 50/F | Cy/TBI | SP | $1.5 \times 10^8$ | 7 | none | 24 | 4 | 17 | No |
| G | 38/F | Cy/TBI | SP | $3 \times 10^7$ | 6 | IL-2 | 42 | 2 | 7 | No |

Cy-Cyclophosphamide, 1400 mg/m$^2$ × 2 days; TBI- fractionated total body irradiation 1400 cGy in 7 fractions over 4 days; SP- stable phase disease; NA- response not achieved