Graft-versus-Leukemia Target Antigens in Chronic Myelogenous Leukemia Are Expressed on Myeloid Progenitor Cells

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Abstract

Purpose: Donor lymphocyte infusion (DLI) reliably induces durable remission in 75% to 80% of patients with relapsed chronic myelogenous leukemia (CML) following allogeneic bone marrow transplantation. We previously reported the identification of a high titer–specific immunoglobulin G response against two novel leukemia-associated antigens, CML28 and CML66, which correlated with immune-induced remission. The present studies characterize expression of CML28 and CML66 in primary hematopoietic tissues.

Experimental Design: Specific monoclonal antibodies to CML28 and CML66 were developed and used to detect antigen expression in leukemia cell lines and primary leukemia tissue on Western blot and immunohistochemistry. Expression patterns were confirmed by antigen-specific real-time PCR.

Results: Both CML28 and CML66 were highly expressed in leukemic blasts from patients with acute myelogenous leukemia and CML blast crisis but barely detectable in normal bone marrow, normal peripheral blood, or leukemic cells from patients with stable-phase CML. In contrast, purified CD34+ progenitors from normal individuals and patients with stable-phase CML expressed high levels of CML28 and CML66 transcript and protein. Immunohistochemical staining for CML66 confirmed rare staining of myeloid precursors in normal marrow and diffuse staining of myeloblastic cells in acute myelogenous leukemia and blast crisis CML marrows.

Conclusions: The expression patterns of CML28 and CML66 are strikingly similar and suggest that antigen expression may play a role in shaping the post-DLI antibody repertoire. The CD34+ restricted pattern of expression of CML28 and CML66 is particularly relevant in light of the notion that DLI likely exerts its curative effect by targeting antigens present in self-renewing malignant progenitor populations in CML.

The immune response induced by donor lymphocyte infusion (DLI) presents a unique opportunity to identify the components of an effective antitumor response. In patients with chronic myelogenous leukemia (CML) who have relapsed after bone marrow transplant, infusion of donor lymphocytes induces durable remission in 70% to 80% of patients (1, 2). Whereas the clinical efficacy of DLI has been established, the mechanism by which DLI exerts its effect remains to be elucidated. One approach to understanding the biology underlying the graft-versus-leukemia effect of DLI is to define the antigenic targets of the DLI response. We previously showed that antigen expression may play a role in shaping the post-DLI antibody repertoire. The CD34+ restricted pattern of expression of CML28 and CML66 is particularly relevant in light of the notion that DLI likely exerts its curative effect by targeting antigens present in self-renewing malignant progenitor populations in CML.

The present studies were undertaken to define the expression pattern of these novel antigens. Both CML28 and CML66 are clearly expressed in several different tumor cell types. However, time of cytogenetic remission, suggesting that B cell–defined antigens may be relevant to the antitumor response. By using serum from DLI responders to screen a CML cDNA expression library, we isolated a panel of 13 candidate tumor-associated antigens (3), including RBP-Jκ, RAFTK, CML28, and CML66. Each of these antigens was found to elicit a specific and high-titer antibody response in the recipient, appearing only after infusion of donor lymphocytes and temporally correlated with the elimination of leukemia cells in vivo (4, 5). To identify the determinants of immunogenicity of our candidate CML-associated antigens, we initiated a series of studies to investigate their molecular and expression characteristics and have used CML28 and CML66 as model antigens. CML28 is a known autoantigen that is a component of the human exosome involved in 3' RNA processing (6–8). CML66 seems well conserved across species, but its function remains undefined. By Northern blot analysis, we previously found CML28 and CML66 to be only rarely expressed in normal tissue and highly expressed in many primary tumors and tumor cell lines (4, 5). Moreover, CML28- and CML66-specific antibody responses were identified in patients with a variety of cancers. These attributes make both CML28 and CML66 attractive as potential targets of antigen-specific immunotherapy.
because we originally identified antibody responses against both antigens in a patient with CML, the studies presented herein focus on their expression in cells of myeloid origin. By Western blotting, quantitative PCR, and immunohistochemistry, we show that both CML28 and CML66 are abundantly expressed in progenitor cells but not in terminally differentiated cells of myeloid origin. These findings suggest that DLI effectively stimulates antitumor activity directed against the malignant stem cell clone that defines CML, thus generating its curative effect.

Materials and Methods

Patient samples. Patient serum, cells, and bone marrow were obtained at various times before and after DLI in patients enrolled on a clinical trial of CD4+ DLI for treatment of relapse after allogeneic bone marrow transplant (9). Samples were also obtained from patients with hematologic malignancies, enrolled on other Institutional Review Board–approved clinical trials at the Dana-Farber Cancer Institute, Boston, MA.

Generation of 22F, a CML66-specific monoclonal antibody. CML66-GST fusion protein was purified as previously described (3). Monoclonal antibody to CML66 (22F) was generated by injecting mice with serial immunizations consisting of 50, 25, and 25 μg of purified recombinant protein in 2-week intervals in the presence of Freund’s Adjuvant. The mice were bled 10 days after the last immunization. Upon first screening of the hybridoma clones, 215 clones were positive for CML66, based on testing the antibody against CML66-GST and FAK-GST by ELISA (to control for nonspecific glutathione S-transferase (GST) binding). On secondary screening of hybridomas to CML66, 34 clones were still positive. Of these, three were subcloned, and one (igGl, κ) was expanded based on Western blotting specificities, and antibody from this clone was subsequently column purified using a Protein A column. This monoclonal antibody was used at 1:100 dilution for Western blotting.

Western blotting. Whole cell lysate was generated from various tumor cell lines or from patient samples by lysis with radioimmunoprecipitation assay buffer [1% NP40, 0.5% deoxycholate, 0.1% SDS, 125 mM/1 sodium chloride, 50 mM/1 HEPES (pH 7.4)] in the presence of protease and phosphatase inhibitors. Lysates (20-30 μg per lane) were subjected to protein gel electrophoresis using 8% to 16% SDS-PAGE with Tris-glycine buffer and transferred onto nitrocellulose filters in 20% methanol in Tris-glycine buffer. Protein bands were visualized as previously described (3). Antibody to β-actin (Sigma, St. Louis, MO) was used as a control to ensure equal loading of lanes.

Immunoprecipitation. Lysates were prepared as above with radioimmunoprecipitation assay buffer. Volumes equivalent to 1,000 μg total protein were diluted in radioimmunoprecipitation assay buffer to a concentration of 1 mg/mL. Diluted lysates were incubated for 1 hour at 4°C with 30 μL 50% Protein G Sepharose (4 Fast Flow; Amersham Pharmacia, Piscataway, NJ) bead slurry to minimize non-specific binding. The precleared lysates were then incubated with 25 μL CML66-specific monoclonal antibody for 3 hours at 4°C. Subsequently, 50 μL 50% Protein G bead slurry were added and incubated for 1 hour. The beads were submitted to two high-stringency washes with radioimmunoprecipitation assay buffer and three low-stringency washes with PBS and resuspended in SDS loading buffer. Finally, the beads were denatured at 95°C and samples were loaded on an 8% to 16% SDS-PAGE gel with Tris-glycine buffer and submitted to Western blotting as described above. A Pyk2-specific monoclonal antibody (BD Signal Transduction Laboratories, San Jose, CA) was used as a negative control antibody for immunoprecipitation experiments.

Isolation of purified cell populations. To isolate CD34+ mononuclear cells derived from bone marrow or peripheral blood of normal donors or patients, an enrichment step was done using the Rosette-Sep bone marrow progenitor reagent, according to manufacturer’s recommendations (Rosette-Sep, Stemcell Technologies, Vancouver, British Columbia, Canada). This enriched population was subsequently stained with anti-CD34-PE antibody (Coulter, Hialeah, FL) for 15 to 20 minutes on ice, washed × 2 in PBS, and isolated by CD34+ magnetic beads (Miltenyi, Auburn, CA). The isolated CD34+ populations were confirmed to be 95% to 99% CD34+ by flow cytometry. Monocytes were purified by isolating the buffy coat layer of a leukopack following sheep RBC rosetting. Dendritic cells were generated by cytokine stimulation and maturation from blood mononuclear monocytes, using standard methods (10). Lysates from these cell populations were generated by lysis with radioimmunoprecipitation assay buffer.

Generation of total RNA. Total cellular RNA was isolated from PBMC or BM by lysis with TRIzol reagent (Invitrogen, Carlsbad, CA). Genomic DNA was sheared by repeatedly forcing lysate through an 18-gauge needle. After addition of chloroform and centrifugation, the aqueous phase was collected and mixed with 100% ethanol and loaded onto an SV spin column assembly (SV Total RNA Isolation System kit; Promega, Madison, WI). RNA bound to the column resin was washed twice with SV wash buffer, centrifuged to dry resin, and eluted with DEPC-H2O. Samples were stored at −80°C until used.

Real-time PCR. Quantitative real-time PCR was used to determine the number of CML28 and CML66 transcripts in patient samples. RNA was generated from patient cells and cell lines as described above. One step cDNA generation and transcript amplification were undertaken per manufacturer’s recommendations (One-Step Taqman, Applied Biosystems, Foster City, CA). CML66-specific primers and probe have been previously described (5). CML28-specific primers and probe were designed and validated, the sequences of which are shown in Table 1. Each 50 μL of reaction mixture contained 0.1 μg RNA and the following concentrations of other components: 1× Taqman buffer A (Perkin-Elmer, Boston MA); 3 mM/L MgCl2; 300 nM/L each primer; 100 nM probe; 200 mM/L dATP, dCTP, dGTP; 400 mM/L dUTP; 17 units/LUNG; and 2 units AmpliTaq DNA polymerase (Perkin-Elmer). cDNA was generated at 48°C for 30 minutes followed by PCR using the following conditions: One cycle of denaturation (95°C for 10 minutes) was followed by 40 cycles of amplification (95°C for 15 seconds, 60°C for 1 minute). PCR was done in a spectrophotometric thermal cycler (ABI PRISM 7700, Applied Biosystems) that measures the independent fluorescent spectrum of each well in a 96-well plate during thermal cycling. A series of standard dilutions of RNA derived from K562 cells (a CML cell line) were used to generate a standard curve. Each patient RNA sample was run in duplicate in a 96-well format along with the dilution series of the K562 RNA. The same samples were also run in duplicate on the same plate with established primers and probes for glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase copy number served as a control for both the quality and amount of RNA in the sample.

Immunohistochemistry. Immunohistochemistry with anti-CML66 monoclonal antibody was done using 5-μm-thick Zenker’s acetic acid-fixed, paraffin-embedded bone marrow tissue sections by standard immunohistochemical methods. Briefly, slides were deparaffinized and pretreated with 1.0 mM/L EDTA (pH 8.0: Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) as per manufacturer’s instructions followed by washing in distilled water. All further steps were done at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity, followed by a 1:5 dilution of goat serum in 50 mM/L Tris-Cl (pH 7.4) for 20 minutes to block nonspecific binding sites. Monoclonal anti-human CML66 antibody was applied at a 1:50 dilution in 50 mM/L Tris-Cl (pH 7.4) with 3% goat serum for 1 hour. Slides were washed in 50 mM/L Tris-Cl, 0.05% Tween 20 (pH 7.4), and goat anti-mouse horseradish peroxidase– conjugated antibody (Envision detection kit, DAKO) was applied for 30 minutes. After further washing, immunoperoxidase staining was
developed using a diamino benzidine chromogen kit (DAKO) per the manufacturer. Finally, slides were placed in a chromogenic-enhancing solution (Zymed) for 5 minutes at room temperature and counterstained with hematoxylin.

**Results**

The **22F monoclonal antibody specifically recognizes CML66 antigen.** Previous work using Northern blot analysis indicated that CML28 and CML66 are highly expressed in a variety of tumor cell lines but not in the majority of normal tissues, including hematopoietic tissues (4, 5). Because these antigens were originally identified based on antibody recognition of the antigen target in a CML patient (patient A), the current studies have focused on characterization of CML28 and CML66 protein expression in myeloid lineage cells. To this end, monoclonal antibodies to CML28 and CML66 were generated. 21F, a CML28-specific monoclonal antibody, has been previously described (4). 22F, a CML66-specific murine monoclonal antibody, was generated in a similar fashion after immunization with purified recombinant CML66-GST fusion protein. CML66 has been previously found to generate two possible transcript products, CML66-L and CML66-S (11). By ELISA, using overlapping peptides to CML66 as coating antigen, the peptide epitope recognized by 22F was mapped to residues 520 to 543 of CML66, near the COOH-terminal of the protein (data not shown). Thus, 22F monoclonal antibody recognizes both of the previously identified CML66 transcript products.

To confirm that 22F correctly identifies the same antigen as sera from patient A, a series of immunoprecipitation studies were done. Lysate from normal bone marrow, stable-phase CML, acute myelogenous leukemia (AML), and HeLa cells were immunoprecipitated with 22F, and Western blotted against patient A sera, at 1:100 dilution, or against 22F. As shown in Fig. 1A, identical bands are identified when the immunoprecipitated lysate was blotted against either patient A sera or 22F thus confirming the specificity of our monoclonal antibody for the CML66 antigen. Moreover, immunoprecipitation of lysate generated from the CML cell line, K562, with post-DLI patient sera, followed by Western blotting against 22F similarly revealed a single band of 66 kDa consistent with CML66 (Fig. 1B). CML66 was identified by Western blot of K562 lysate immunoprecipitated with 22F but not by Western blot of the K562 lysate immunoprecipitated with an antibody against an alternate CML-associated antigen, RAFTK (Fig. 1C). RAFTK is abundantly present in K562 cells and was also previously identified as a DLI-associated CML antigen by serologic screening (3).

CML28 and CML66 are highly expressed in primary myeloid leukemia cells. Using 22F, Western blotting experiments were done to examine the expression of CML66 in whole cell lysates generated from peripheral blood and marrow of normal donors and peripheral blood of leukemic patients. Representative examples of these Western blot experiments are shown in Fig. 2. We found that 11 of 14 AML samples expressed CML66 at high levels. Among CML patient samples, minimal to low expression was found in stable-phase CML samples, but high expression was observed in three of three blast crisis samples. In addition,

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**Table 1. CML28-specific Taqman primers**

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>CML28</td>
<td>5′-CCCGGCTCTTCTCTGCAAC-3′</td>
</tr>
<tr>
<td>CML28</td>
<td>5′-ACAAGCCACACTGAATGTA-3′</td>
</tr>
<tr>
<td>Probe</td>
<td>5′-6FAM-CAG GCA GCC CAA TCT TCG GCC T-TAMRA-3′</td>
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**Fig. 1.** The 22F monoclonal antibody specifically recognizes CML66 antigen. A, immunoprecipitation of whole cell lysates from HeLa cells, normal bone marrow (BM), PBMC of patients with stable-phase CML (CML-SP) and AML with 22F, and subsequently Western blotted with sera of patient A, revealed a single band of 66 kDa consistent with CML66. B, immunoprecipitation of K562 lysates with or without patient A sera and Western blotted with 22F, similarly revealed a single 66-kDa band consistent with CML66. C, immunoprecipitation of K562 lysates with either the 22F monoclonal antibody or a control antibody (anti-RAFTK) and Western blotted with either of the same antibodies.
CML66 was highly expressed in two of two myeloid leukemia cell lines (K562 and Mo7E). In contrast, normal PBMC and normal bone marrow showed absent or minimally detectable levels of CML66 protein. Further isolation and purification of a variety of mature myeloid lineage cell populations, including monocytes and dendritic cells, showed only low or absent levels of expression. The expression pattern of CML28, by Western blotting with 21F, was strikingly similar. Details of CML28 expression in primary myeloid leukemia cells and cell lines have been previously reported (4). Like CML66, CML28 was only minimally expressed in mature myeloid lineage cell populations.

**Immunohistochemical staining of CML66 in leukemic versus normal bone marrow.** To determine which hematopoietic cell populations express CML66, a series of immunohistochemistry studies were undertaken on a variety of normal and leukemic bone marrow samples using 22F. 21F was nonreactive in Zenker's fixed paraffin-embedded tissue; thus, these studies were not done for CML28. Consistent with our Western blotting results, we found that nine of nine bone marrows collected from patients with AML were strongly positive for expression of CML66 by immunohistochemistry. A representative example of the staining is shown in Fig. 3B and shows diffuse strong positivity in cells with morphologic characteristics of myeloblasts. In contrast, when normal bone marrow was stained, as shown in Fig. 3A, the only significant positive staining resided in nests of myeloid precursor populations. Normal lymphoid and erythroid populations were not stained. These studies indicate that CML66 is preferentially expressed in undifferentiated myeloid lineage cells.

**CML66 expression in CML bone marrow correlates with stage of disease.** Although an antibody response to CML66 was detected at the time of immune therapy-induced remission in patient A, who had stable-phase CML, Western blotting for CML66 in whole cell lysates from peripheral blood of a number of stable-phase CML patients showed relatively little expression of this protein. The composition of the myeloid elements in peripheral blood of patients with CML can range from predominantly mature granulocytic to early blastic populations, depending on the stage of disease. These characteristics of CML, taken together with the aforementioned observations of preferential expression of CML66 in the early myeloid lineage

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (kDa)</th>
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<tr>
<td>CML28</td>
<td>28</td>
</tr>
<tr>
<td>CML66</td>
<td>66</td>
</tr>
<tr>
<td>β-actin</td>
<td>40</td>
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Fig. 2. CML66 is highly expressed in leukemia cells but at only low levels in normal cells. Whole cell lysates, generated from BM from normal donors (NL BM) or PBMC from patients with a variety of hematologic malignancies, including AML, stable-phase CML (CML-SP), and blast crisis CML (CML-BC), as well as from purified monocytes and dendritic cells isolated from two to three different normal donors were loaded at a concentration of 20 μg per lane on a 10% to 12% SDS-PAGE gel and electrophoresed. Lysates generated from myeloid cell lines K562 and Mo7E were also loaded. The protein gel was blotted against monoclonal antibody specific to CML66 (66 kDa). β-Actin was used as a control to ensure equal loading per lane.

Fig. 3. **A**, immunohistochemical staining of a normal bone marrow with an anti-CML66 monoclonal. **B**, immunohistochemical staining of bone marrow derived from a patient with AML with an anti-CML66 monoclonal.
cells, led us to query whether CML66 is preferentially expressed in the early, undifferentiated progenitor compartment in CML as well. Immunohistochemical staining of CML66 expression in patients with CML was therefore studied on serial bone marrow samples, collected from a series of CML patients through the course of therapy.

Figure 4A-C shows a representative example of these experiments and shows the immunohistochemical staining that was done on a series of three marrows from patient AA. Figure 4A is a marrow sample collected from patient AA within 1 year after diagnosis, following discontinuation of treatment with hydroxyurea and IFN-α, and just before initiating treatment with the selective tyrosine kinase inhibitor, imatinib mesylate (Gleevec). This marrow shows significant CML66 staining in clusters of early-appearing myeloid cells, interspersed with areas of absent staining. Three months later, patient AA achieved a major cytogenetic response to imatinib, and the second marrow, shown in Fig. 4B, was obtained at this time. Immunohistochemical analysis at this time shows that the number of cells expressing CML66 decreased dramatically. However, as shown in Fig. 4C, patient AA’s disease evolved to blast crisis some months later. At this time, the marrow showed diffuse and high level expression of CML66, similar to the pattern of expression in marrows derived from patients with AML.

**CML28 and CML66 expression in bone marrow is restricted to undifferentiated myeloid cells.** To confirm that CML28 and CML66 expression within myeloid lineage cells resides predominately in the early undifferentiated population, CD34+ and CD34−/C0 cell fractions from peripheral blood of patients with stable-phase CML and from bone marrow of normal volunteers were isolated, and analyzed by Western blotting. As shown in Fig. 5, in both normal BM and CML peripheral blood, both CML28 and CML66 were only minimally detected in the CD34− fraction but easily detected in the CD34+ cell populations. Equal amounts of protein were loaded per lane, as shown by the equal expression of β-actin in each lysate. These results show that CML28 and CML66 expression in myeloid lineage cells is restricted to an early progenitor population.

Whereas both CML28 and CML66 showed CD34− restricted expression by Western blot, they differed in their relative expression in normal versus leukemic CD34+ cells. Whereas CML-derived CD34+ cells seemed to have slightly higher CML28 expression on Western blot than normal CD34+ cells, the levels of CML66 expression between these two CD34+ cell populations were equivalent. Similar results were observed upon examination of CML28 and CML66 RNA expression by quantitative PCR. As shown in Fig. 6, the mean expression levels of CML28 and CML66 per microgram of RNA were high in PBMC derived from patients with AML (686 and 746 copies per μg RNA, respectively; n = 7) and with CML-BC (myeloid; 691 and 679 copies per μg RNA, respectively; n = 4), both groups whose peripheral blood counts consisted of >90% CD34+ blasts. In contrast, mean CML28 and CML66 expression in total RNA from normal BM (221 and 259 copies per μg RNA, respectively; n = 4), PBMC (102 and 203 copies per μg RNA, respectively; n = 5), and T cells (131 and 174 copies per μg RNA, respectively; n = 5) was ∼3- to 6-fold lower. The mean level of CML28 RNA expression in normal bone marrow derived CD34+ isolated cells (319 copies per μg RNA; n = 4) was lower than in AML blasts and CML-BC cells but elevated.
relative to the other normal cell populations. In contrast, for CML66, the mean copy number of transcripts in normal bone marrow derived CD34+ isolated cells (930 copies per μg RNA; n = 4) was as high as in AML blasts and CML-BC cells. Thus, we observed that whereas the total mass of marrow myeloid progenitor cells expressing CML66 is greater in patients with leukemia than in normal volunteers (by immunohistochemistry), CML66 expression, on a per cell basis, is similar between normal and malignant CD34+ cells. The overall striking similarity in expression patterns between CML66 and CML28 suggest that abundant expression in CD34+ cells is a common feature of antigenic targets of the DLI response.

**Discussion**

Genetic studies done >20 years ago showed that the malignant clone in CML is a pluripotent hematopoietic stem cell, capable of differentiating into myeloid cells, monocytes, erythrocytes, and platelets (12–18). Phenotypic characteristics of human hematopoietic stem cells, precursors, and progenitor populations have been identified (19), and in general, these subpopulations can be identified based on CD34+ expression in combination with present or absent expression of additional immunophenotypic markers. In CML, the property of self-renewal has been recently identified within the stem cell population of CML patients with chronic-phase disease and within the stem cell and myeloid progenitor populations in patients with blast crisis (20). These findings have significant therapeutic implications and strongly suggest that any curative therapy for CML requires the ability to eradicate these leukemic stem cell populations (21).

Hematopoietic stem cell transplantation and DLI can be curative for CML. The clinical effectiveness of DLI implies that at least some target antigens of graft versus leukemia must be expressed on the malignant CML stem or progenitor cell, but to date, only rare tumor-specific or allo-specific candidate antigens have been described that meet these expression characteristics (22–25). Our current study expands the repertoire of immunogenic DLI-associated antigens that are expressed on myeloid progenitor cells. Herein, we describe a series of studies in which expression of two DLI-associated antigens, CML28 and CML66, were analyzed in primary hematopoietic tissue derived from CML patients and compared with normal volunteers. Using specific monoclonal antibodies for these novel antigens, Western blotting studies showed low expression of both antigens in normal bone marrow, PBMC, and mature myeloid populations, and high expression in AML and advanced CML. Immunohistochemical analysis of CML66 expression on normal and leukemic marrow samples revealed that the extent of CML66 expression seemed concordant with number of leukemic blasts and with the patients' clinical course. Finally, by isolating CD34+ populations from PBMC of CML patients and normal BM and by using Western blotting and quantitative PCR, we show that expression of both antigens was enriched in the CD34+, and not in the CD34− population.

Our expression studies yielded a number of unexpected findings. First, we observed that the overall expression patterns of CML28 and CML66 are strikingly similar. That two of eight antigens identified by serologic screening of a CML cDNA library using sera derived from the same patient exhibited the same expression pattern (3) suggests that high expression within the early myeloid progenitor cell population may be a common characteristic of DLI targeted antigens. Further exploration of this hypothesis is under way, as we further characterize the expression patterns of our other identified candidate antigens.

Second, although both CML28 and CML66 showed CD34+ enriched expression by Western blotting, we found that these antigens differed from each other in their expression on a per cell basis in normal and leukemic CD34+ cells. Our Western blotting and quantitative PCR studies support that CML28 seems more highly expressed in leukemic than in normal CD34+ cells. This expression pattern has been seen in other promising immunotherapy targets such as survivin and proteinase 3 (22, 26–30). Overexpression may be a source of immunogenicity, by virtue of lowering the threshold at which an immune response is initiated, as has been suggested by other investigators (31, 32). In contrast, whereas our immunohistochemistry and Western blotting studies show that leukemic marrow and peripheral blood contain far greater numbers of cells expressing abundant levels of CML66 compared with normal marrow, CML66 seems equally expressed in both normal and malignant CD34+ cells on a per cell basis. This pattern of expression has been observed in other tumor-associated antigens that are under investigation as immunotherapy targets, such as the Wilms tumor gene (WT1). Similar to CML66, WT1 has been reported to be highly expressed in
leukemia and expressed at low levels in total bone marrow lysate (25) but to show equal expression in CD34+ isolated cells derived from normal marrow and AML (33–35). Despite these findings, WT1-specific CTLs have been generated that can selectively eliminate leukemic CD34+ progenitor cells (36). Thus, even with similar levels of expression between normal and leukemic CD34+ cells, as we have shown for CML66, this antigen may still be a suitable immunotherapy target. Possibly, differential peptide processing and presentation between leukemic compared with normal CD34+ cells could result in preferential cytotoxic T-cell recognition of malignant hematopoietic progenitors (37–39). Whereas robust cellular expression is a prerequisite for antigen presentation, clearly, whether an antigen becomes an available target for effector activity by CTL is more complicated. Future studies will examine whether CML66 and CML28-specific CTLs can be generated that specifically target leukemic CD34+ cells.

In instances where antibody responses post-DLI are directed against cell surface antigens, B-cell responses can have direct antitumor effects. This has recently been shown in patients with myeloma, where some DLI responders have been found to develop antibodies specific for BCMA, a cell surface antigen which is selectively expressed on myeloma cells and mature B cells (40, 41). In these patients, post-DLI serum was able to induce complement-mediated lysis and antibody-dependent cell cytotoxicity of transfected cells and primary myeloma cells expressing BCMA. In this scenario, antibody responses to cell surface BCMA may directly contribute to tumor rejection in vivo. In contrast, CML66 and CML28 are both intracellular antigens; thus, antibodies targeting these antigens would not participate in direct antibody-induced elimination of leukemia cells by complement or antibody-dependent cytotoxicity. On the other hand, the remission inducing effect of DLI is likely primarily mediated through T-cell effector function. Smit et al. previously showed the increased frequency of T cell responses against leukemic CD34+ progenitors following DLI, concurrent with clinical response (42). Despite the insight that increased T-cell reactivity against leukemic progenitors are strongly linked to the curative effect of DLI, very few studies have identified the target antigens on the malignant myeloid progenitors against which these T-cell responses are directed. Whereas it is possible that antigen-specific antibodies are generated only as a result of graft-versus-leukemia-associated killing, we speculate that it is also possible that that high titer antibodies to CML66 and CML28 could augment anti-leukemic T-cell responses against these CML progenitor cell antigens. In both murine and human systems, antigen-antibody complexes have been shown to facilitate cross-presentation of target antigens through a FcyR-mediated pathway in dendritic cells and the stimulation of CD8+ T-cell responses (43–45). If future T-cell studies confirm the specificity of CML28- and CML66-specific CTLs for leukemic progenitors and together with the current expression studies, they will provide solid rationale for developing these antigens as targets for specific immunotherapy of CML.

References


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