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### Article

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# Detection of a potent humoral response associated with immune-induced remission of chronic myelogenous leukemia

Catherine J. Wu,<sup>1,3</sup> Xiao-Feng Yang,<sup>1,3</sup> Stephen McLaughlin,<sup>1</sup> Donna Neuberg,<sup>2</sup> Christine Canning,<sup>1</sup> Brady Stein,<sup>1</sup> Edwin P. Alyea,<sup>1,3</sup> Robert J. Soiffer,<sup>1,3</sup> Glenn Dranoff,<sup>1,3</sup> and Jerome Ritz<sup>1,3</sup>

<sup>1</sup>Center for Hematologic Oncology, and

<sup>2</sup>Department of Biostatistical Science, Dana-Farber Cancer Institute, and

<sup>3</sup>Department of Medicine, and Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

Address correspondence to: Jerome Ritz, Dana-Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA. Phone: (617) 632-3465; Fax: (617) 632-5167; E-mail: jerome\_ritz@dfci.harvard.edu.

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The effectiveness of donor-lymphocyte infusion (DLI) for treatment of relapsed chronic myelogenous leukemia (CML) after allogeneic bone marrow transplantation is a clear demonstration of the graft-versus-leukemia (GVL) effect. T cells are critical mediators of GVL, but the antigenic targets of this response are unknown. To determine whether patients who respond to DLI also develop B-cell immunity to CML-associated antigens, we analyzed sera from three patients with relapsed CML who achieved a complete molecular remission after infusion of donor T cells. Sera from these individuals recognized 13 distinct gene products represented in a CML-derived cDNA library. Two proteins, J $\kappa$ -recombination signal-binding protein (RBP-J $\kappa$ ) and related adhesion focal tyrosine kinase (RAFTK), were recognized by sera from three of 19 DLI responders. None of these antigens were recognized by sera from healthy donors or patients with chronic graft-versus-host disease. Four gene products were recognized by sera from CML patients treated with hydroxyurea and nine were detected by sera from CML patients who responded to IFN- $\alpha$ . Antibody titers specific for RAFTK, but not for RBP-J $\kappa$ , were found to be temporally associated with the response to DLI. These results demonstrate that patients who respond to DLI generate potent antibody responses to CML-associated antigens, suggesting the development of coordinated T- and B-cell immunity. The characterization of B cell-defined antigens may help identify clinically relevant targets of the GVL response in vivo.

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## Introduction

Allogeneic bone marrow transplantation (BMT) has become widely accepted as standard curative therapy for chronic myelogenous leukemia (CML) (1, 2). The therapeutic effect of BMT derives partially from the eradication of leukemia cells by high-dose chemotherapy and radiation. However, several clinical observations provide convincing evidence that donor immune responses also contribute substantially to the elimination of residual CML cells and the subsequent success of BMT. First, graft-versus-host disease (GVHD), a major complication of allogeneic BMT in which immunocompetent donor cells damage host tissue, is associated with decreased incidence of leukemia relapse (3, 4). Second, recipients of syngeneic stem cells experience increased risk of CML relapse despite administration of similar ablative regimens (4). Third, depletion of T cells from donor marrow to reduce the risk of GVHD results in a significantly increased risk of disease relapse (4, 5). Finally, infusion of donor lymphocytes without additional therapy can successfully induce remission in 75–80% of patients with relapsed

CML after allogeneic BMT (6, 7). Taken together, these clinical observations provide compelling evidence that donor T cells play an important role in mediating a graft-versus-leukemia (GVL) response as well as GVHD after allogeneic BMT. However, the mechanisms whereby donor T lymphocytes exert an antileukemic response are unknown, and the target antigens of this response have not been well defined. Given the reproducible efficacy of donor lymphocyte infusion (DLI), the treatment of CML with donor lymphocytes represents a powerful clinical system for defining the mechanisms and targets of effective tumor immunity in vivo.

Although donor T cells are mediators of both GVHD and GVL after allogeneic BMT, studies in murine models suggest that these immune responses may be distinguished in some systems. Clinical observations also suggest that these two responses may be distinct in some patients. For example, it has been noted that some patients who respond to DLI do not also develop GVHD. In particular, patients who receive CD8-depleted donor lymphocytes appear to have a reduced risk of GVHD while maintaining a high degree of GVL activity

(8, 9). These observations suggest that at least some of the target antigens of GVL are distinct from those that also cause GVHD, but in most patients, the cellular antigens that are the targets of these immune responses remain poorly defined. To identify the target antigens recognized by donor T cells after allogeneic BMT, other investigators have established alloreactive T-cell clones *in vitro* and have used different methods to determine the antigenic specificity of these cells. These approaches have been successful in determining the peptide specificity of several alloreactive T-cell clones, but only limited numbers of target antigens have been identified thus far (10, 11). Most of the peptide antigens identified using these approaches have been demonstrated to represent genetic polymorphisms that distinguish donor from recipient and have been categorized as minor histocompatibility antigens (mHA) (12–14).

Alternate approaches that exploit the B-cell immune response have also been used to identify target antigens of immune responses *in vivo*. Two decades ago, Old demonstrated the reactivity of patient serum with autologous tumor cells, thus providing evidence for a specific humoral response to human cancer cells (15). Autoantibodies have also been used to identify disease-specific molecular targets in autoimmune and paraneoplastic disease (16, 17). In 1995, Sahin et al. described a method for serological identification of antigens by recombinant expression cloning (SEREX) to identify tumor-associated genes (18). The SEREX approach has proven to be a powerful method for identification of new cancer antigens. Since the initial description of this methodology, more than 580 tumor antigens have been identified, and approximately one-third of these antigenic targets represent novel genes (<http://www.licr.org/SEREX.html>; ref. 19). Importantly, many antigens identified previously as T-cell targets have also been shown to be targets of B-cell immunity, and conversely, antigens originally identified because of antibody reactivity have subsequently been shown to be targets of T-cell responses *in vivo* (20). This approach has also been applied to CML where Ling et al. used autologous serum from a patient with untreated CML to screen a cDNA-expression library derived from that same patient to define CML-associated antigens (21). Eight gene products were identified in this study, including two previously unreported fusion gene products. Of note, *bcr-abl* was not identified as a target antigen using autologous patient serum.

In 1994 we initiated a clinical trial using CD4<sup>+</sup> donor T-cell infusions in patients with relapsed leukemia after BMT and achieved clinical response rates in CML comparable to previous experience with unfractionated DLI (9). During this trial, increased numbers of peripheral B cells were observed in several patients at the time of clinical response. Bone marrow biopsies frequently revealed persistent lymphocytosis and plasmacytosis. These findings suggest that a potent B-cell response developed after DLI. We therefore hypothesized that serum from patients responding to DLI would contain antibodies directed against leukemia-associated antigens and

adapted the SEREX approach to identify antigens associated with the GVL response after DLI. Sera from patients responding to DLI were used to screen a CML-derived cDNA expression library to identify CML-derived gene products associated with an effective antileukemic response. These experiments demonstrate the presence of a potent humoral immune response after donor lymphocyte infusion that is directed at a series of CML-associated antigens. This approach may provide a novel method for identifying target antigens of the GVL response *in vivo*.

## Methods

*Preparation of cell and plasma samples.* Heparinized blood samples were obtained from patients and normal donors enrolled on clinical research protocols at the Dana-Farber Cancer Institute. All clinical protocols were approved by the Human Subjects Protection Committee of the Dana-Farber Cancer Institute. The majority of samples were obtained from patients with relapsed CML enrolled on clinical trials evaluating the toxicity and efficacy of infusion of defined doses of CD4<sup>+</sup> donor lymphocytes (9). Samples were also obtained from patients undergoing allogeneic transplantation with CD6<sup>+</sup> T cell-depleted marrow from matched related donors (22) and from patients with chronic GVHD after transplantation with unmodified marrow and conventional immune suppressive therapy. Additionally, samples were obtained from patients with CML who were receiving hydroxyurea, from four CML patients who had achieved a major cytogenetic response after treatment with IFN- $\alpha$ , and from seven patients who were refractory to IFN- $\alpha$  treatment. PBMC from normal donors and patients 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 by removal of the plasma layer after centrifugation of whole blood and cryopreserved at  $-80^{\circ}\text{C}$  until the time of analysis.

*CML cDNA library construction and screening.* mRNA was extracted separately from PBMC from three patients with CML, one with accelerated-phase and two with stable-phase disease, using standard methods (FastTrack Kit; Invitrogen, Carlsbad, California, USA) and pooled to create a representational CML-expression library. A total of 5  $\mu\text{g}$  of mRNA derived from CML cells was used to construct the library. cDNA inserts were directionally cloned into a  $\lambda$ ZAPII bacteriophage expression vector (ZAP-cDNA Gigapack III Gold Cloning Kit; Stratagene, La Jolla, California, USA). Analysis of 20 individual phage plaques of the library revealed cDNA inserts ranging in size from 0.5 to 3 kb.

To screen the library, XL1-Blue MRF' *Escherichia coli* (Stratagene) were transfected with recombinant phage, plated on agar at  $5 \times 10^4$  plaques per 150-mm Petri dish, and cultured at  $37^{\circ}\text{C}$  for 5 hours. Expression of recombinant proteins was induced by incubation with isopropyl  $\beta$ -D-thiogalactoside-treated (IPTG-treated) nitrocellulose membranes for an additional 3.5 hours at

37°C. Filters were subsequently washed in TBST (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, 0.05% wt/vol Tween 20, pH 8.0) to remove excess agar and blocked overnight with 1% wt/vol nonfat dry milk in TBS. The filters were then incubated overnight with post-DLI patient serum, diluted at 1:500 in TBST. This serum was preabsorbed against phage lysate and the *E. coli* strain to minimize nonspecific antibody binding. Specific binding of antibody to recombinant proteins expressed on the lytic plaques was detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Labs, West Grove, Pennsylvania, USA) diluted at 1:2000 in TBST. Visualization of the antigen-antibody complex was accomplished by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) (Promega, Madison, Wisconsin, USA).

cDNA inserts from positive clones were isolated by excision of phagemids. The cDNA inserts were then sequenced with T3 and T7 primers (Dana-Farber Cancer Institute Molecular Biology Core Facility).

**DNA sequence analysis.** Sequence alignments were performed using the GenBank databases to determine whether these were related or identical to known genes.

**Phage-plate assay.** Phages from positive clones of interest were mixed with nonreactive phage of the cDNA library as internal negative controls at a ratio of 1:10. This mix was used to transfect 200  $\mu$ L of XL1-Blue MRF' *E. coli* bacteria. The bacteria and phage were plated onto NZY agar plates and grown at 37°C for 5 hours. Expression of recombinant proteins was induced by incubation with IPTG-treated nitrocellulose membranes for an additional 3.5 hours at 37°C. Filters were subsequently washed in TBST to remove excess agar and blocked overnight with 1% wt/vol nonfat dry milk in TBS. The filters were then incubated overnight with patient serum preabsorbed against phage lysate and the *E. coli* strain to minimize nonspecific antibody binding, and diluted at 1:200–1:500 in TBST. Specific binding of antibody to recombinant proteins expressed on the lytic plaques was detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG antibody diluted at 1:2000 in TBST. Visualization of the antigen-antibody complex was performed by staining with BCIP/NBT.

**Purification of recombinant proteins.** We purified a bacterially expressed fusion protein consisting of glutathione-S-transferase (GST) and the COOH-terminus (amino acids 681–1009) of human RAFTK cDNA subcloned into a pGEX-2T expression vector (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), as described previously (23) (gift of R. Salgia, Dana-Farber Cancer Institute). The induction of recombinant protein synthesis and subsequent purification of proteins was performed by elution from Sepharose GST beads (Amersham Pharmacia Biotech). Correct size and specificity of the expressed product were confirmed by Western blotting using mouse anti-human anti-PYK2 antibody (Transduction Laboratories, Franklin Lakes, New

Jersey, USA). Purification of a bacterially expressed fusion protein consisting of GST and our CML library-derived clone C57 was similarly achieved. The expression plasmid for this J $\kappa$ -recombination signal-binding protein (RBP-J $\kappa$ ) homologue was made by PCR-based subcloning of the C57 phagemid into vector pGEX-3x (Amersham Pharmacia Biotech). Appropriate size and specificity of the expressed product was confirmed by Western blotting using polyclonal rabbit anti-human RBP-J $\kappa$  sera (gift of E. Kieff, Brigham and Women's Hospital). Recombinant GST protein (without fusion protein) was purified by standard techniques and used as a control.

**ELISA assay.** ELISA plates (VWR Scientific, South Plainfield, New Jersey, USA) were coated with 50  $\mu$ L of purified recombinant protein at 5  $\mu$ g/mL in coating buffer (PBS + 0.05% sodium azide) overnight at 4°C. Plates were washed with PBS with 0.05% Triton and blocked overnight at 4°C with 200  $\mu$ L/well 2% nonfat milk with 0.05% Triton. Patient sera (50  $\mu$ L/well) was added to a final dilution of 1:200 and incubated at room temperature for 3 hours. After several washes, the plates were incubated with 50  $\mu$ L/well of alkaline phosphatase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Labs), diluted 1:1000, for 1 hour at room temperature. Finally, the plates were washed and incubated with 75  $\mu$ L/well of PNPP substrate (Sigma, St. Louis, Missouri, USA) for 25 minutes at room temperature, and OD (405) was immediately read (Spectramax 190 Microplate Reader; Molecular Devices, Sunnyvale, California, USA). In addition to using RAFTK-GST and C57-GST fusion proteins as coating antigens, each sera sample was tested against GST alone as a control for nonspecific binding. The ratio of the OD RAFTK-GST/OD GST or OD C57-GST/OD GST was used to determine the degree of specific reactivity above background. An OD ratio two standard deviations above the mean ratio of OD (test antigen)/OD GST of 13 normal donor sera (1.44 for C57 and 1.32 for RAFTK) was interpreted as positive. Specificity of each positive sample was examined by testing reactivity after preincubation with test antigen. Confirmed positive samples had decreased test-antigen reactivity on ELISA compared with nonpreincubated sera or sera preincubated with irrelevant antigen or GST. Samples that were not confirmed to be specifically positive for the test antigen in this fashion were excluded from further analysis.

## Results

**Screening of CML cDNA library with post-DLI patient sera.** A CML cDNA library was constructed and the library was screened using banked serum (diluted 1:500) from three patients (designated patients A, B, and C) 1 year after receiving DLI for relapsed CML (Table 1). All three patients demonstrated prompt cytogenetic responses to therapy, with disappearance of Ph-positive metaphases at 2–5 months after DLI infusion, and absence of detectable *bcr-abl* transcript using RT-PCR

**Table 1**

Clinical characteristics of three DLI responders whose sera was used for library screening

Patient	Age at BMT/Sex	Treatment before BMT	Months from BMT to cytogenetic relapse	Treatment before DLI	Months from BMT to DLI	Months from DLI to complete cytogenetic response	Months from DLI to complete molecular response
A	25/F	Hydroxyurea	11	IFN	54	5	12
B	47/M	Prior allogeneic BMT hydroxyurea	12	IL-2, IFN	25	3	8
C	38/F	Hydroxyurea	6	IL-2	24	2	7

by 1 year after DLI (Table 1). None of the patients developed acute or chronic GVHD after DLI. Initial screening with each patient sera was performed to determine if reactivity to the phage library antigens was present. In each case, 30–76 reactive clones were identified with the initial screen. Once the clones were plaque purified, a second level of screening was undertaken to determine whether clones were recognized by individual patient sera collected before the original BMT, before DLI (relapse), and/or 1 year after DLI (Table 2). After testing sera from all three patients, 11 clones were reactive with sera at all time points and were excluded from further analysis. Eight clones were not reactive with pre-BMT sera but were reactive with sera collected both before and after DLI. These clones may represent antigens associated with alloimmunity, but were not further examined in this report. Finally, 33 clones demonstrated selective reactivity with post-DLI sera. These clones were most likely to be associated with the GVL effect of DLI and were further characterized.

**DNA sequence analysis of GVL-associated CML clones.** After screening to identify those clones with selective reactivity with post-DLI sera, 14, 11, and eight clones were identified from screens A, B, and C, respectively. The cDNA inserts from each clone were isolated and restriction enzyme digest, and sequence analysis indicated that the clones from screens A, B, and C corresponded to eight, three, and four distinct gene products, respectively. GenBank database analysis revealed these clones to have close homology to 13 distinct gene products listed in Table 3. Eleven of these clones represent known genes, and two represent unknown genes. The known genes appear to represent a variety of intracellular proteins involved in different cellular functions, including cell-cycle progression, differentiation, and signaling (Table 3). Some gene products were multiply represented among the clones, suggesting a high level of representation in the CML cDNA expression library. For example, RAFTK represented nine out of 33 positive clones. Eleven genes were identified by individual patient sera, but two of the 13 gene products (RAFTK and RBP-J $\kappa$ ) were identified independently by sera from two patients. Note that none of the isolated antigens are identical to the gene products identified by the autologous screening of a CML library by Ling et al. (21). GenBank analysis revealed that clones encoding known genes encompass 20–100% of the opening reading frames (ORFs) of

the corresponding genes. Three clones contain complete coding regions (thymosin  $\beta$ -4, phorbolin-1-related protein, and defensin); all others constitute the COOH-terminal portions of the genes. Several of the clones contain single nucleotide differences compared with reported GenBank sequences. Other clones have sequence insertions or deletions. For example, the RBP-J $\kappa$ -related clone C57 contains a 350-bp insertion, resulting in theoretical premature termination of the ORF.

**Time course of reactivity to GVL-associated gene products after DLI.** The phage plate assay was used to define the pattern of antibody reactivity against the 13 identified target antigens in the three patients in which antibodies were initially detected. Sera obtained at various times before and after DLI were used at 1:500 dilution. As summarized in Table 4, antibody reactivity to each of the 13 gene products was demonstrated in post-DLI sera but not before DLI or after BMT. Although the initial library screens were performed using 1-year post-DLI sera, we observed reactivity to nine antigens as early as 3 months after DLI. Whereas the phage-plate assay is not a quantitative test, intensity of reactivity to these antigens appeared to increase with sera obtained 6–12 months after DLI, suggesting rising titers of antibody to these antigens at these time points. Remarkably, antibodies against five of the gene products (phorbolin-1-related protein, 7-60 protein, KIAA0530 protein, thymosin  $\beta$ -4, and RBP-J $\kappa$ ) remained present in sera obtained 4 years after DLI.

**Identification of antibodies against GVL-associated antigens in other patients with CML.** To assess whether sera from other DLI responders recognize this same panel of gene

**Table 2**

Number of cDNA clones identified by post-DLI sera (1:500 dilution) from patients A, B, and C

		Patient A	Patient B	Patient C
Pre-BMT	+			
Pre-DLI	+	4	3	4
Post-DLI	+			
Pre-BMT	-			
Pre-DLI	+	2	6	0
Post-DLI	+			
Pre-BMT	-			
Pre-DLI	-	14	11	8
Post-DLI	+			

Clones were categorized into three groups: positive with pre-BMT, pre-DLI, and post-DLI sera; positive only after BMT; and positive only after DLI.

**Table 3**

cDNA clones identified by serologic screening of CML library

Clone	Patient A	Patient B	Patient C	GenBank sequence homology	CML clone sequence	Comparison between GenBank and library sequences
C9	1			T54 protein <sup>A</sup>	79% ORF	95% identity
C12	1			Phorbolin-1-related protein (52)	100% ORF	99% identity
C13	1			CHD protein (53)	23% ORF	95% identity
C53, C8c, C21	3			7-60 protein <sup>B</sup>	27% ORF	99% identity
C29, C41	2			KIAA0530 protein (54)	55% ORF	99% identity
C57, W2, W5, W11, W14	1	4		RBP-J $\kappa$ (55)	50% ORF	78% identity
C3, C25, C33, C42	4			Unknown gene no.1		
C59	1			Unknown gene no. 2		
W18		1		Thymosin- $\beta$ 4 (56)	100% ORF	93% identity
R30			1	Defensin 1 (57)	100% ORF	93% identity
W4, W9, W19		3		ANG2 mRNA <sup>C</sup>	27% ORF	91% identity
R18			1	RBBP-5 (58)	84% ORF	99% identity
R5, R7, R8, R21, R22, R28, W16, W17, W24		3	6	RAFTK (39)	20% ORF	99% identity

<sup>A</sup>GenBank accession no. U66359. <sup>B</sup>GenBank accession no. NM-007346. <sup>C</sup>GenBank accession no. AF024631. RBBP-5, retinoblastoma binding protein 5.

products, we used the phage-plate assay to test 1-year post-DLI sera (at 1:200 dilution) from 16 other patients with CML who had complete cytogenetic and molecular responses after DLI. As summarized in Table 5, one additional DLI responder recognized RAFTK and another DLI responder recognized RBP-J $\kappa$ . None of the gene products were recognized by sera from ten normal donors or from ten patients with extensive chronic GVHD after allogeneic BMT. When sera from 11 patients with CML receiving hydroxyurea as primary therapy were tested, most did not recognize any of the 13 gene products. However, sera from three CML patients recognized RBP-J $\kappa$ , and three other patients individually recognized KIAA0530, chromodomain helicase DNA-binding (CHD) protein, and defensin. We also examined sera from ten CML patients who had undergone CD6<sup>+</sup> T cell-depleted BMT but who had not relapsed or developed GVHD. Within this group of ten patients, none of the antigens were recognized, with the exception of one patient whose post-BMT sera recognized RBP-J $\kappa$ . Sera from 11 patients undergoing T cell-depleted BMT for other hematologic malignancies (four acute myelogenous leukemia [AML], three acute lymphoblastic leukemia [ALL], one myelodysplastic syndrome [MDS], two Hodgkin's disease [HD], and one non-Hodgkin's lymphoma [NHL]) also did not recognize these gene products, suggesting that the development of an antibody response is not related to the myeloablative conditioning regimen.

In addition to patients who had undergone BMT or DLI, we also examined sera from four

patients with CML who had major cytogenetic responses after treatment with IFN- $\alpha$ . Remarkably, nine of the 13 gene products were recognized by at least one of the four patients. Antibodies against RBP-J $\kappa$  were detected in three of the four IFN- $\alpha$  responders. In contrast, of seven IFN- $\alpha$ -refractory patients tested, only one had a detectable antibody response, which was directed against CHD protein.

*Quantitation of antibodies specific for RAFTK and RBP-J $\kappa$ .* Since RAFTK and RBP-J $\kappa$  (C57) were recognized by sera from multiple DLI responders and CML patients, we developed ELISA assays to quantitate the humoral immune response to these antigens. Recombinant COOH-terminus RAFTK-GST and C57-GST fusion proteins were purified and used as ELISA coating antigens. Recombinant GST protein alone was used as a negative control for specific antigen recognition. All sera were tested at a dilution of 1:200. As shown in Figure 1, none of the 13 normal donor sera exhibited reactivity against C57 (RBP-J $\kappa$ ) or RAFTK compared with reactivity to

**Table 4**

Time course of reactivity before and after donor lymphocyte infusion using phage plate assay

Gene product	Pre-BMT	Pre-DLI	3 mo after DLI	6 mo after DLI	1 yr after DLI	4 yr after DLI
T54 protein	-	-	+	++	++	-
Phorbolin	-	-	+	++	+	++
CHD protein	-	-	-	-	+	-
7-60 protein	-	-	+	++	++	+
KIAA0530 protein	-	-	-	+	++	++
RBP-J $\kappa$	-	-	+	+	+	+
Unknown gene no. 1	-	-	+	++	++	-
Unknown gene no. 2	-	-	+	++	+	-
ANG2	-	-	+	+	+	-
Thymosin $\beta$ -4	-	-	-	-	+	+
RAFTK	-	-	+	+	+	-
RBBP5	-	-	-	-	+	NA
Human defensin 1	-	-	+	+	+	NA

+, positive detectable reactivity by phage-plate assay; ++, increased intensity of reactivity detected after color development.

**Table 5**

Serum reactivity with leukemia-associated antigens using phage-plate assay.

Gene product	DLI responders <sup>A</sup>	Normal	Chronic GVHD	CML Hydroxyurea	CML TCD BMT	Other TCD BMT (AML, ALL, HD, NHL, MDS)	CML IFN- $\alpha$ responders	CML IFN- $\alpha$ refractory
T54 protein	1/19	0/10	0/10	0/11	0/10	0/11	0/4	0/7
Phorbolin	1/19	0/10	0/10	0/11	0/10	0/11	0/4	0/7
CHD protein	1/19	0/10	0/10	1/11	0/10	0/11	0/4	1/7
7-60 protein	1/19	0/10	0/10	0/11	0/10	0/11	1/4	0/7
KIAA0530 protein	1/19	0/10	0/10	1/11	1/10	0/11	1/4	0/7
RBP-J $\kappa$	3/19	0/10	0/10	3/11	0/10	0/11	3/4	0/7
Unknown gene no. 1	1/19	0/10	0/10	0/11	0/10	0/11	1/4	0/7
Unknown gene no. 2	1/19	0/10	0/10	0/11	0/10	0/11	2/4	0/7
Thymosin $\beta$ 4	1/19	0/10	0/10	0/11	0/10	0/11	1/4	0/7
Defensin 1	1/19	0/10	0/10	1/11	0/10	0/11	1/4	0/7
ANG2	1/19	0/10	0/10	0/11	0/10	0/11	0/4	0/7
RBBP-5	1/19	0/10	0/10	0/11	0/10	0/11	1/4	0/7
RAFTK	3/19	0/10	0/10	0/11	0/10	0/11	1/4	0/7

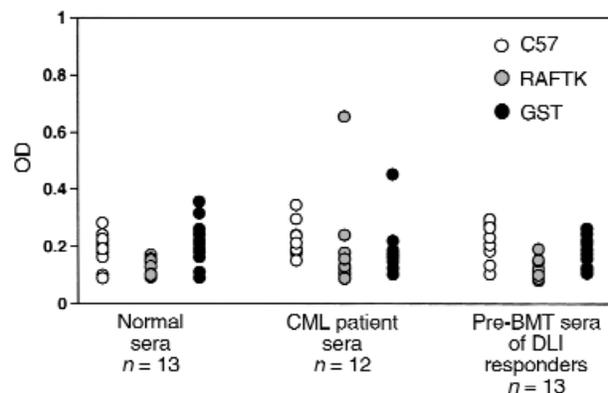
<sup>A</sup>All DLI responders had previously undergone allogeneic T cell-depleted BMT. Included in the 19 patients are the three patients whose sera were used to screen the CML cDNA expression library (patients A, B, and C). HD, Hodgkin's disease; NHL, non-Hodgkin's lymphoma.

GST. In addition to sera from ten hydroxyurea-treated CML patients that were tested by phage-plate assay, we examined sera from two other similarly treated CML patients. As in the phage-plate assay, none were reactive to either antigen, except one of the two additional patients who displayed significant antibody reactivity against RAFTK. Finally, when we examined sera from 13 DLI responders obtained before BMT, no reactivity to either test antigen was seen.

The ELISA assays for RAFTK and RBP-J $\kappa$  were also used to confirm that our DLI sera contained high-titer antibody specific for these two antigens. Positive ELISA reactivity against C57 (RBP-J $\kappa$ ) was maintained at serum dilutions of 1:80,000 to 1:160,000, and reactivity against RAFTK was maintained at dilutions of 1:25,000 (Table 6). Preincubation of patient sera with purified recombinant C57 (RBP-J $\kappa$ ) or RAFTK protein specifically inhibited ELISA reactivity, but preincubation with GST alone or with irrelevant antigen did not reduce reactivity measured by ELISA. The results shown in Figure 2 confirm the specificity of the antigen-antibody interaction in the ELISA assay.

*Detection of anti-RAFTK antibody responses in DLI responders.* Of the 19 DLI responders, three patients (designated patients B, C, and D) demonstrated a significant antibody response to this antigen by ELISA (Figure 3a). These were the same three patients whose sera were identified as RAFTK-reactive by phage-plate assay. Sera from both patients B and C demonstrated no significant reactivity to RAFTK before BMT and DLI, but developed rising antibody titers beginning at 3–4 months after DLI, which peaked at 6–12 months (to 1:25,000 titer) after DLI. Subsequent to 1 year after DLI, the antibody titer waned and fell to baseline by 4 years after DLI. Patient D developed a similar pattern of antibody reactivity to RAFTK, but with a lower peak titer response (1:3,200 titer). It is interesting that patient D received immunosuppressive therapy for 1 year after DLI. All three patients achieved cytogenetic remission by 2–3 months after DLI and molecular remission (*bcr/abl*-PCR negative) by 8 months after DLI.

*Detection of anti-C57 (RBP-J $\kappa$ ) antibody responses in DLI responders.* Using the ELISA assay, we detected four DLI responders with specific reactivity against C57, one more than originally identified by phage-plate assay. The pattern of antibody response against C57 was distinctly different from that against RAFTK (Figure 3b). Two of the four responders (patients A and B) developed low levels of specific reactivity after transplant and before DLI. This reactivity was detected by ELISA, but not by phage-plate assay, suggesting the increased sensitivity of the former test. On review of their clinical histories, we found that both patients A and B were successfully treated with IFN- $\alpha$  after CML relapse after BMT and before DLI. The antibody response against C57 further increased after DLI, peaking at maximal titers of 1:80,000 to 1:160,000 at 3–6 months after DLI. Consistent with the phage-plate data, antibody titers to this antigen are sustained for several years after DLI. Two other DLI responders (patient E and F) did not

**Figure 1**

Quantitative serum reactivity against RAFTK and C57(RBP-J $\kappa$ ) measured using ELISA. Normal sera ( $n = 13$ ), sera from CML patients ( $n = 12$ ) receiving hydroxyurea, and pre-BMT sera from DLI responders ( $n = 13$ ) demonstrate no reactivity to RAFTK or C57 (RBP-J $\kappa$ ) compared with reactivity against GST negative control. One patient with CML demonstrated significant reactivity against RAFTK. All sera were diluted at 1:200.

**Table 6**  
Maximal antibody titers to RAFTK and C57 (RBP-Jκ) using ELISA

Reactivity to C57		Reactivity to RAFTK	
Time point	Maximal titer	Time point	Maximal titer
Patient A, 6 mo after DLI	1:80,000	Patient B, 4 mo after DLI	1:25,000
Patient B, 6 mo after DLI	1:160,000	Patient C, 12 mo after DLI	1:25,000

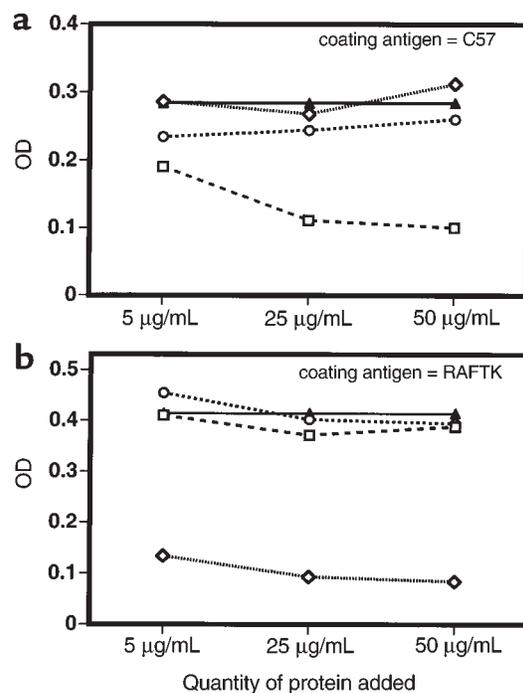
have detectable reactivity before BMT or DLI, but developed antibodies to C57 after DLI. These individuals developed maximal titers of 1:160,000 and 1:50,000, respectively, 12 to 24 months after DLI. Patient E achieved a cytogenetic remission 5 months after DLI, but molecular remission was not obtained until 17 months after DLI. Patient F achieved prompt cytogenetic and molecular remissions after DLI but received immunosuppressive therapy for GVHD until 2 years after DLI.

### Discussion

Previous studies have established the utility of the SEREX method for identification of relevant target antigens of tumor immunity and autoimmunity (24, 25). This method has several advantages over conventional methods based entirely on T-cell recognition of undefined antigens. Unlike T-cell receptors, immunoglobulins are able to bind specifically to target antigens in soluble form and without requirements for antigen presentation or MHC restriction. It is therefore easier to clone the target genes directly for B cell-defined antigens. Recent work has demonstrated the close correlation between the humoral and cellular responses to SEREX-defined tumor antigens (20). Thus, once B cell-defined antigens have been identified, it is possible to determine which of these gene products are also targets of T-cell immunity and to further characterize the T-cell response in selected patients. Though this approach has been successful in the characterization of autologous immune responses, we did not know whether this could be applied to patients after allogeneic BMT where B-cell function is generally considered to be deficient and where antibody responses against allogeneic target antigens have not been described. Nevertheless, we observed increased numbers of circulating B cells and plasma-cell infiltrates in some DLI responders, suggesting that effective elimination of tumor cells in the GVL response represented a coordinated immune response that included both B- and T-cell immunity to relevant target antigens. We therefore applied the SEREX method to the identification of target antigens in patients with well-documented GVL and have been able to demonstrate potent and long-lived antibody responses to a number of diverse gene products. These observations support our hypothesis that the GVL response represents a complex and coordinated immune response against a variety of cellular antigens and sug-

gests that the further characterization of B-cell responses may rapidly expand our ability to identify the relevant target antigens of this clinically effective anti-tumor response *in vivo*.

After the screening of our CML-expression library using sera obtained 1 year after DLI from three patients who achieved complete remission after treatment, we identified 33 clones that were only reactive with post-DLI sera. DNA sequence analysis demonstrated that these clones represented 13 distinct gene products. Why potent antibody responses developed against this particular group of antigens remains unclear. In other patients with cancer, immunogenicity of tumor-associated antigens can result from overexpression in tumor cells after increased gene transcription (26) or DNA amplification (27). Alternatively, chromosomal rearrangements (28, 29) or gene mutations (30) can cause the presentation of novel immunogenic epitopes. Some mutations do not change the epitope itself, but affect posttranslation modification and protein trafficking, leading to more effective immune presentation of the tumor antigen (31). In several instances (e.g., RAFTK, RBP-Jκ, and unknown gene 1) we identified multiple cDNA clones during library screening, sug-

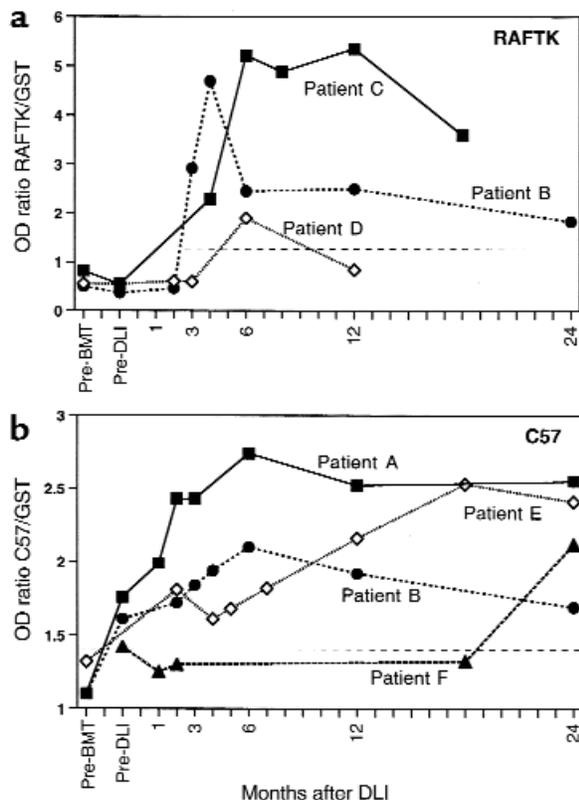


**Figure 2**  
Competitive ELISA to confirm specificity of the antigen-antibody interaction in patient sera. Patient A serum was reactive with C57 (RBP-Jκ) and patient B serum was reactive with RAFTK. Patient serum samples were preincubated for 3 hours at room temperature with increasing amounts of RAFTK-GST (open diamonds, dotted line), C57-GST (open squares, dashed line), GST alone (open circles, dashed line), or with PBS (filled triangles, straight line), and then tested using ELISA for reactivity with C57 (a) or with RAFTK (b). Only preincubation with specific target protein resulted in inhibition of reactivity measured by ELISA.

gesting the overexpression of these genes by CML cells used to create the cDNA expression library. Examination of the DNA sequence of the genes identified by DLI sera also demonstrated several base-pair differences in the sequence of some clones compared with the published GenBank sequence of homologous genes. Larger insertions were also noted in some clones. It is therefore possible that both increased expression and specific mutations may contribute to the immunogenicity of the gene products identified by these experiments. Further analysis will be necessary to determine which of these factors is important for each of the gene products we have identified in this initial characterization of the antibody response associated with DLI.

Our analysis of reactivity to our panel of antigens suggests that the immune response we detect by SEREX is primarily directed against leukemia-associated rather than allogeneic target antigens. None of the antigens were recognized by sera from normal donors, from patients with chronic GVHD, or from patients undergoing T cell-depleted BMT for various hematologic malignancies other than CML. In contrast, antibodies to several of these gene products were frequently detected in sera from patients with CML who had not undergone allogeneic BMT. This is most evident in our examination of sera from four patients who had major responses to IFN- $\alpha$  where antibodies specific for nine of the 13 antigens were present in sera from at least one of these patients. Despite having high titers of specific antibodies, none of the patients whose sera were used for screening developed evidence of autoimmune disease. This contrasts with immune responses to solid tumors, such as melanoma, where antitumor responses are typically associated with autoimmune vitiligo, because immunity has developed against antigens expressed by both tumor cells and normal tissue (32).

Even though the antibody responses we detected in patients after DLI do not appear to be directed against targets selectively expressed on allogeneic recipient cells, it remains possible that these B-cell responses may reflect an initial response directed against T cell-defined alloantigens. In the majority of antigens we identified, the DNA sequences contain several single-nucleotide substitutions compared with GenBank sequences. These differences may represent single-nucleotide polymorphisms (SNP) that may distinguish recipient from donor cells (33). Such SNP may result in immunogenic mHA and may be responsible for the initial targeting of these gene products for immune recognition. Several mHA that are selectively expressed in hematopoietic cells have been described previously (34–36). It is likely that many other such antigens exist and that they will play an important role in both the GVL and GVHD responses (37, 38). Further analysis of the gene products we have identified and the possible SNPs contained within these genes will be necessary to determine if these may also represent mHA and whether this contributes to the immunogenicity of these gene products after DLI.



**Figure 3** Quantitative antibody reactivity to RAFTK (a) and C57(RBP-J $\kappa$ ) (b) was measured using ELISA in responders at various times before and after DLI. Serial serum samples from three DLI responders (1:200 dilution) were tested for specific reactivity against RAFTK, and serial serum samples from four DLI responders were tested for reactivity against C57(RBP-J $\kappa$ ). The dashed line in each part indicates the upper limit of the negative control (two SD above the mean ratio of OD [test antigen]/OD GST for 13 normal donor sera).

After initial characterization of the antibody response to 13 distinct gene products, we further examined the humoral immune response to two antigens, RBP-J $\kappa$  and RAFTK, since they were most widely recognized by CML patient sera. Using purified recombinant proteins, ELISA assays were established to specifically quantitate the antibody response at different times before and after DLI in patients who recognized these antigens. Generally, results using ELISA were consistent with our phage-plate data. All sera that were reactive using phage-plate assay were also positive using ELISA. In two patients, antibodies to RBP-J $\kappa$  were detected before DLI by using ELISA but not by using the phage-plate assay. This discrepancy likely reflects the higher sensitivity of the ELISA for detecting specific antibody in patient serum.

RAFTK is a member of the focal adhesion kinase family of proteins, normally expressed in brain and hematopoietic cells. RAFTK appears to link calcium, integrin, chemokine or stress-activated signaling and cytokine-mediated pathways to nuclear-activating proteins involved in cellular proliferation (39). RAFTK also acts as a kinase for the cytoskeletal protein paxillin (40),

which is believed to be an important substrate and binding site for *bcr-abl*. Several hematopoietic cell functions, including NK-cell activation (41), integrin-dependent activation of primary human neutrophils by TNF, and T-cell signaling have been shown to involve RAFTK signaling. More recently, RAFTK has also been implicated in signaling pathways in cancers, such as Kaposi's sarcoma (42) and multiple myeloma (43). Although previous reports have suggested that phosphorylation of RAFTK occurs in CML (40), the role of this gene product as a potential tumor-associated antigen has not been explored previously.

RBP-J $\kappa$  [also mammalian CBF-1, Su(H) in *Drosophila*] is the primary downstream target of the highly conserved Notch-signaling pathway, which is important in regulation of cellular differentiation in many organs, including the hematopoietic system (44). In the absence of binding to the intracellular Notch receptor, RBP-J $\kappa$  acts as a transcriptional repressor (45). RBP-J $\kappa$  has been implicated in Epstein-Barr virus (EBV) transformation, in which binding to EBNA2 unmasks its repression domain and thereby causes transcriptional activation (46, 47). In the absence of Notch signaling, RBP-J $\kappa$  has been found to repress activity of NF $\kappa$ B2 (48) as well as IL-6 gene transcription (49). This activity implies that RBP-J $\kappa$  may also play a significant role in immune modulation. Although Notch signaling is known to play an important role in hematopoietic differentiation, the activity of RBP-J $\kappa$  in CML and the possible role of this gene product as a tumor-associated antigen have not been examined.

When serial samples after DLI were tested using ELISA, the antibody responses against RAFTK and RBP-J $\kappa$  were markedly different. In all three individuals with reactivity to RAFTK, the antibody response to RAFTK occurred only after DLI and first became detectable around the time of response to DLI. After complete remission had been achieved, antibody titers to RAFTK gradually subsided. In contrast, two patients were found to have low but detectable antibody responses to RBP-J $\kappa$  before DLI. Antibody reactivity to RBP-J $\kappa$  increased after DLI, but this increase did not appear to be temporally associated with clinical responses. Unlike RAFTK, anti-RBP-J $\kappa$  titers were sustained for long periods up to 5 years after DLI (data not shown). Two other patients developed a delayed response to RBP-J $\kappa$ , only detectable 1 to 2 years after DLI. Interestingly, one of these patients achieved delayed molecular remission; the other received immunosuppressive agents for 2 years after DLI. Whereas the antibody response to RBP-J $\kappa$  correlates less well with DLI, the possibility that it may be associated with GVL after BMT cannot be excluded. Moreover, its sustained pattern of reactivity may reflect an immune-protective role for anti-RBP-J $\kappa$  antibodies or the presence of continued stimulus for the antibody response. Future studies characterizing the T-cell response to these antigens will help us better understand to what degree they are correlated with the anti-tumor response initiated by DLI.

Although all patients we examined appeared to develop high-titer antibody responses to a variety of leukemia-associated antigens, our studies do not demonstrate that the humoral immune response directly contributes to the antileukemic effect of DLI. In some murine models of tumor immunity, autoantibodies provide a primary effector mechanism or are required to sustain an effective response initiated by T cells (50, 51). In other murine models, the cytotoxic CD8-mediated T-cell response is the most critical element of effective tumor immunity. In such cases, the appearance of high-titer antibody may simply reflect the presence of a T-cell response and release of intracellular proteins after leukemia cell death. As a group, all of the antigens we identified are intracellular, consistent with the findings of other investigators who have employed SEREX to identify tumor-associated antigens (25). However, having identified a series of diverse B-cell antigens, further studies can now be directed to examine these gene products as potential targets of T-cell immunity after DLI. In this context, it is evident that even though several patients developed responses to the same antigens, most DLI responders did not have detectable reactivity to any of the antigens identified in our initial three DLI responders. This observation suggests that the number of leukemia-associated antigens may be quite diverse. This may reflect the high degree of diversity of human HLA as well as the large number of potential antigenic targets. Further studies to determine whether the leukemia-associated antigens we have identified in this report are also targets of T-cell immunity after DLI will be critical to developing a better understanding of the immunologic mechanisms responsible for effective tumor immunity in these patients. These studies will also establish whether the analysis of B cell-defined antigens will provide a method for the rapid identification of the immunogenic targets as well as the diversity and complexity of this response.

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