

## Graft-versus-Leukemia Antigen CML66 Elicits Coordinated B-Cell and T-Cell Immunity after Donor Lymphocyte Infusion

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### Abstract

**Purpose:** The target antigens of graft-versus-leukemia that are tumor associated are incompletely characterized.

**Experimental Design:** We examined responses developing against CML66, an immunogenic antigen preferentially expressed in myeloid progenitor cells identified from a patient with chronic myelogenous leukemia who attained long-lived remission following CD4+ donor lymphocyte infusion (DLI).

**Results:** From this patient, CML66-reactive CD8+ T-cell clones were detected against an endogenously presented HLA-B\*4403-restricted epitope (HDVDALLW). Neither CML66-specific antibody nor T-cell responses were detectable in peripheral blood before DLI. However, by 1 month after DLI, CD8+ T cells were present in peripheral blood and at 10-fold higher frequency in marrow. Subsequently, plasma antibody to CML66 developed in association with disease remission. Donor-derived CML66-reactive T cells were detected at low levels *in vivo* in marrow before DLI by ELISpot and by a nested PCR-based assay to detect clonotypic T-cell receptor sequences but not in blood of the patient pre-DLI nor of the graft donor.

**Conclusions:** CD4+ DLI results in rapid expansion of preexisting marrow-resident leukemia-specific donor CD8+ T cells, followed by a cascade of antigen-specific immune responses detectable in blood. Our single-antigen analysis thus shows that durable posttransplant tumor immunity is directed in part against nonpolymorphic overexpressed leukemia antigens that elicit coordinated cellular and humoral immunity.

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Whereas much progress has been recently made in the development of potent immunotherapy reagents, the generation of consistent tumor immunity through defined antigen vaccination remains elusive (1–3). In this context, dissecting the mechanisms of response in instances of clinically evident antitumor immunity can be highly instructive. Allogeneic hematopoietic stem cell transplant (HSCT) for leukemia remains the most established example of human immunity, whose curative effect

depends on mounting donor-derived immune responses, called the graft-versus-leukemia (GvL) effect (4). The results of donor lymphocyte infusion (DLI) for treating relapsed chronic myelogenous leukemia (CML) further illustrate the potency of GvL (5, 6). In this procedure, lymphocytes derived from the donor are infused—typically without further radiation or chemotherapy—and consistently result in durable remissions in 70% to 80% CML patients.

One approach to dissecting immunity following DLI and HSCT is through identification of antigens targeted by these therapies. Studies in patients and mouse models have firmly established alloantigens as an important antigen class targeted by GvL, but mounting evidence suggests that tumor-associated antigens elicit at least part of this response (7–12). We therefore focused our analysis on immune responses directed against CML66. This CML-associated antigen was previously identified to elicit high-titer antibodies in a CD4+DLI-treated individual, who developed prompt GvL without clinically evident graft-versus-host disease. CML66 is not an alloantigen, as no sequence differences within CML66 between donor and recipient were discovered (13). On the other hand, CML66 is highly expressed in leukemia cells and particularly in CD34+ myeloid progenitor cells (14).

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### Translational Relevance

A robust example of long-lasting human tumor immunity is the excellent result of donor lymphocyte infusion (DLI) for the treatment of relapsed chronic myeloid leukemia, but the underlying mechanisms of this complex immune response are poorly characterized. We identify and track CD8+ T-cell immunity against a graft-versus-leukemia (GvL) antigen, CML66, within the context of DLI-induced remission. Our results indicate that CD4+ DLI results in the rapid expansion of preexisting marrow-resident leukemia-specific CD8+ T cells, followed by a cascade of peripheral B and T antigen-specific immune responses. Effective GvL thus comprises at least partly of immunity against nonpolymorphic B-cell and T-cell epitopes. The discovery of leukemia antigens within the context of therapeutic response, such as CML66, provides important tools for dissecting effective tumor immunity; insights gained from these studies provide a rational path for devising more specific and less toxic immunotherapy.

In the current study, we analyzed immunity against CML66 as a means to characterize the kinetics of developing GvL responses. Because CD4+ T-cell responses can aid generation of both antigen-specific antibody and CD8+ T-cell responses, we queried whether development of potent B-cell responses indicated presence of antigen-specific cytolytic T-cell immunity. From peripheral blood, we isolated CD8+ cytotoxic T-cell clones directed against a CML66-derived HLA-B4403-restricted 8-mer peptide. We found expansion of functional CML66-specific T cells in peripheral blood and marrow as early as 1 month after DLI, in close temporal association with antibody responses. Intriguingly, donor-derived CML66-specific T cells that were not present in T cells of the original donor were detected in the marrow even before DLI. Together, our analysis of the responses to this single leukemia-associated antigen suggests that DLI-induced GvL is a multicomponent process. This process involves both antigen priming to generate a pool of preexisting leukemia-specific T cells residing in the marrow reservoir before DLI, functional antileukemia cytotoxic T-cell responses in marrow and peripheral blood, together with leukemia-directed antibody responses, and recruitment of new antigen-specific T cells developing after DLI. Our study provides evidence that effective GvL responses comprise long-lived and coordinated immunity against overexpressed leukemia antigens.

### Materials and Methods

**Patient samples.** Heparinized blood, skin biopsies, or marrow aspirates were obtained from patients and normal donors enrolled on clinical research protocols at Dana-Farber

Cancer Institute. All clinical protocols were approved by Dana-Farber Cancer Institute Human Subjects Protection Committee. Peripheral blood mononuclear cells (PBMC) or bone marrow mononuclear cells 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 after centrifugation of whole blood and cryopreserved at  $-80^{\circ}\text{C}$  until use. Primary skin fibroblast cultures were generated from 5-mm diameter skin punch biopsies that were minced into  $<0.5\text{-mm}$  fragments, affixed to culture dishes with fibrin clots, and fed with MCDB106/M199 medium (1:1 v/v; Life Technologies) + 10 ng/mL epidermal growth factor (Millipore) + 0.4  $\mu\text{g/mL}$  hydrocortisone (EMD Biosciences). Fibroblasts that migrated out and divided from these explants were suspended 7 days later with trypsin/EDTA, subcultured in the same medium, and expanded.

**Sources of antigen.** We synthesized 91 overlapping 15-mers to 18-mers (unpurified) encompassing the coding region of CML66 (<http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html>) and a series of 8-mer, 9-mer, and 10-mers ( $>85\%$  purity) to define the peptide epitope of CML66-reactive T cells (New England Peptide). Synthetic peptides encoding the cytomegalovirus (CMV) pp65 (NLVPMVATV) and the Mart1<sub>26-35</sub> epitopes (M26, EAAGIGILTV) were used as controls. Peptides were reconstituted in DMSO (10 mg/mL) and stored at  $-20^{\circ}\text{C}$ . CML66 was cloned into a standard E1-deleted and E3-deleted adenoviral vector and expressed under the control of a bidirectional CMV immediate early gene promoter that expressed green fluorescent protein simultaneously. The Ad/CML66 plasmid was constructed and produced following standard adenoviral vector production protocols (Jeng-Shin Lee and Richard Mulligan; Harvard Gene Therapy Initiative). CML66 was also PCR-cloned into pcDNA3.1 (5' primer, AGCTTTAAGCTTCACCATGGAGG-TGGCGGCTAATTGCCCTAC; 3' primer, ATAGAATCTGCAGTCAATGGTGATGGTGATGATGATTCTCTGTATT-TACTTTTATTA).

**Generation of antigen-presenting cells.** Patient EBV-transformed B-cell lines were generated by incubating PBMC with supernatant from B95-8 Marmoset cells and maintained in RPMI 1640, supplemented with 10% FCS, and antibiotics. For the generation of dendritic cells (DC), CD14+ cells were immunomagnetically isolated (Miltenyi Biotec) per manufacturer's guidelines and cultured in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% human antibody serum (Cellgro), 2 mmol/L glutamine, 50  $\mu\text{g/mL}$  human transferrin (Roche), 5  $\mu\text{g/mL}$  human insulin (Sigma-Aldrich), and 15  $\mu\text{g/mL}$  gentamicin (Invitrogen) in the presence of 50 ng/mL granulocyte macrophage colony-stimulating factor (Genzyme) and 20 ng/mL interleukin-4 (IL-4; R&D Systems). On days 3 and 6, 50% of fresh medium with cytokines was added. On day 7, cells were matured for 48 hours with 1  $\mu\text{g/mL}$  prostaglandin E2 (Sigma-Aldrich),

10 ng/mL IL-1 $\beta$  (BD Bioscience), 1,000 units/mL IL-6 (BD Bioscience), and 10 ng/mL tumor necrosis factor  $\alpha$  (Genzyme). CD40-B cells were generated from PBMC by activation on CD40L-expressing irradiated feeder cells in the presence of IL-4 (R&D Systems) and cyclosporin A (Novartis) as described (15). The MHC class I-deficient immortalized B lymphoblastoid cell line 721.221 was retrovirally infected with individual plasmids encoding the full-length B\*4403, B\*4402, A\*203, A\*3301 alleles (16) and maintained in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, antibiotics, and 1 mmol/L sodium pyruvate. MUTZ-3 cells (DSMZ) were cultured in  $\alpha$ -MEM (Invitrogen) and supplemented with 20% fetal bovine serum and granulocyte macrophage colony-stimulating factor (10 ng/mL; R&D Systems).

**Expression of CML66 in antigen-presenting cells.** CML66 was introduced into DCs by adenoviral transduction. Ad/CML66 viral supernatant was spin-infected (multiplicity of infection = 100; 1,250  $\times$  g at 37  $^{\circ}$ C for 2 hours) onto 0.4  $\times$  10<sup>6</sup> immature DCs, which were seeded in 24-well plates in Iscove's modified Dulbecco's medium supplemented with 10% human sera. Immediately following spin infection, DC maturation cytokines were added. Infected DCs were used 24 to 48 hours later. By flow cytometry, infection routinely generated green fluorescent protein expression in 70% to 90% of antigen-presenting cells (APC). For some experiments, CML66 was introduced into DCs or CD40L-stimulated B cells using RNA nucleofection. For production of CML66 transcript, endotoxin-free CML66/pcDNA3.1 plasmid (Endofree Maxiprep kit, Invitrogen) was linearized with *Xba*I (NE Biolabs), *in vitro* transcribed, and polyadenylated (mMessage mMachine; Ambion). Mart1 transcripts were similarly generated following linearization of pOBT7-Mart1 cDNA (American Type Culture Collection) with *Ssp*I (NE Biolabs). Polyadenylated RNA (2-10  $\mu$ g) was nucleofected into 2 million CD40-B cells in 100  $\mu$ L of PBS/10% HEPES buffer (Program Q-004, Amaxa Nucleofector Device; Lonza, Inc.).

**Cloning of CML66-specific T cells.** Autologous mature DCs (1  $\times$  10<sup>5</sup> per well) were adenovirally transduced to express CML66 and cultured with thawed post-DLI CD3+ T cells (2  $\times$  10<sup>6</sup>/2 mL) with IL-7 (10 ng/mL; Endogen, Inc.) on day 0 and IL-2 (100 IU/mL; Amgen) starting on day 1. Medium was replenished twice weekly with fresh IL-2. Ten days following this single stimulation, CD8+ T cells were immunomagnetically selected (Miltenyi Biotec) and tested by ELISpot against autologous matured DCs pulsed with various pools of CML66-derived overlapping peptides (10  $\mu$ g/mL per peptide). Reactive T cells were expanded using irradiated peptide 66-72-pulsed autologous EBV cells in the presence of 1% phytohemagglutinin and 100 IU/mL of recombinant IL-2. This population was further enriched by selection of cells secreting IFN- $\gamma$  in response to peptide 72-pulsed autologous EBV cells (IFN- $\gamma$  Catch Reagent, Miltenyi Biotec) and cloned by limiting dilution on feeder cells (irradiated allogeneic PBMC and EBV cells, with 100 units/mL recombinant human IL-2 and phytohemagglutinin). Clones displaying

specific anti-CML66 reactivity (see assays below) were further expanded using similar culture conditions.

**Detection of antigen-specific T cells.** Cytolytic assays were done with Europium-labeled target cells (Perkin-Elmer) per manufacturer's directions. Target cells (5,000 per well) were labeled for 30 minutes with Europium, washed extensively, and coincubated at different effector-to-target cell ratios in triplicate for 2 hours, and specific Europium release was measured. ELISpot was done using peptide-pulsed target cells (50,000 per well) coincubated with 200 to 1,000 T-cell clones per well in duplicate in ELISpot plates (Millipore) for 24 hours. IFN- $\gamma$  secretion was detected using capture and detection of antibodies as directed (Mabtech AB) and imaged (ImmunoSpot Series Analyzer; Cellular Technology). To test dependence on class I of T-cell reactivity, ELISpot plates were first coated with APCs in the presence of class I blocking antibody (W6/32) for 2 hours at room temperature before introduction of T cells into the wells. Antigen-specific T-cell reactivity was also detected by IFN- $\gamma$  secretion assay as per manufacturer's recommendations (Miltenyi Biotec), and labeled cells were analyzed by flow cytometry (Beckman-Coulter FC500).

**Chimerism analysis.** Quantitative sequencing of informative single-nucleotide polymorphisms between Patient A and her donor was done as previously described (17). We identified informative polymorphisms that were tested against recipient-derived genomic DNA (extracted from patient fibroblasts) and donor-derived genomic DNA (from donor PBMC) using a panel of single-nucleotide polymorphisms with high minor allele frequency. Once identified, these loci were tested against genomic DNA extracted from immunomagnetically selected (Miltenyi Biotec) marrow-derived CD8+ T cells (Pyrosequencing AB).

**Detection and quantitation of T-cell receptor V $\beta$  clonotypes.** We modified our previous method for T-cell receptor (TCR) spectratyping (18) into a two-step process. First, we identified the dominant V $\beta$  subfamily among the 24 known V $\beta$  subfamilies. We generated five pools of V $\beta$  forward primers (pool 1, V $\beta$  1-5.1; pool 2, V $\beta$  5.2-9; pool 3, V $\beta$  10-13.2; pool 4, V $\beta$  14-19; and pool 5, V $\beta$  20-24; see Supplementary Table S3). RNA extracted from the T-cell clones (QIAamp RNA Blood Mini-kit; Qiagen) was reverse transcribed into cDNA (Superscript, Life Technologies Bethesda Research Laboratories) using random hexamers and PCR-amplified in five separate 20- $\mu$ L volume reactions, each consisting of 10  $\mu$ L of GeneAmp Fast Master Mix (Applied Biosystems), pooled V $\beta$  subfamily forward primers (0.5  $\mu$ mol/L per primer per pool), 0.5  $\mu$ mol/L of reverse primer specific for the TCR constant region (C $\beta$  external primer), and 100 to 200 ng cDNA. Second, T-cell clone-derived cDNA was reamplified with each of the five individual primers contained within a positive pool together with a FAM-conjugated C $\beta$  reverse (internal) primer. For both steps, the amplification conditions were 95  $^{\circ}$ C for 20 seconds; followed by 35 cycles of 95  $^{\circ}$ C for 3 seconds, 55  $^{\circ}$ C for 7 seconds, and 60  $^{\circ}$ C for 30

seconds; and then 60°C for 2 minutes. The amplified fragment was cloned into pCR2.1-TOPO (Invitrogen) and sequenced. Based on this sequence, a quantitative Taqman PCR assay was designed such that a sequence-specific probe was located within the region of junctional diversity (Applied Biosystems). To determine the size distribution of the amplified V $\beta$  subfamily, the fluorescent PCR product was electrophoresed on a 3730 DNA sequencer (Applied Biosystems) and analyzed (Genemapper software, v.4.0; Applied Biosystems).

To detect clonotypic V $\beta$  transcripts directly from patient RNA, nested PCR was done first using the clone-specific V $\beta$  forward primer (0.5  $\mu$ mol/L) and C $\beta$  reverse primer (0.5  $\mu$ mol/L), together with cDNA (4  $\mu$ L) and GeneAmp Fast PCR Master Mix (10  $\mu$ L) in a 20- $\mu$ L reaction volume, using identical amplification conditions as described above. Subsequently, 4  $\mu$ L of this PCR product were amplified with 1  $\mu$ L of the clone CDR3 region-specific primer and probe and 10  $\mu$ L of Taqman Fast Universal PCR Master Mix (Applied Biosystems) in a total volume of 20  $\mu$ L. The PCR amplification conditions were 95°C for 20 minutes  $\times$  1 cycle and 40 cycles of 95°C for 3 seconds followed by 60°C for 30 seconds (7500 Fast Real-Time PCR Cycler; Applied Biosystems). Test transcripts were quantified relative to GAPDH transcripts by calculating  $2^{-(\text{GAPDH } C_t - \text{target } C_t)}$ .

**Peptide ELISA.** Individual or pools of peptides (5  $\mu$ g/mL) were applied to Nunc C96 Maxisorp plates (Fisher) in carbonate buffer (pH 9.6) overnight at 4°C and then blocked in PBS–0.05% Tween with 2% milk. Plasma samples were added to duplicate wells in blocking buffer (1:200 dilution) and incubated for 3 hours at 25°C. Peptide binding of patient immunoglobulin was detected using an alkaline phosphatase-conjugated antihuman IgG secondary antibody (1:1,000 dilution, Jackson ImmunoResearch) and visualized with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich). Absorbance was read at 405 nm. Positive peptide reactivity was defined as signal  $>2$  SD above the mean of 10 normal volunteers.

## Results

**Detection of CD8+ T cells reactive against CML66 in a DLI responder.** We previously discovered potent antibody responses (at titers  $>1:64,000$ ) against the CML-associated protein CML66 that developed in close temporal correlation with disease regression following DLI in Patient A (13). CML66 has unknown function, although residues 268 to 399 are homologous to NUDC, which is involved in nuclear distribution during mitosis (19). Patient A relapsed with CML within 1 year after T cell-depleted myeloablative matched related donor HSCT and was infused with  $3 \times 10^8$  donor CD4+ T cells/kg 4.5 years after transplant, to which she developed prompt cytogenetic remission followed by durable molecular remission ( $>10$  years; Supplementary Table S1). We speculated that such high-titer antibody responses would coordinately develop with an antigen-specific cellular memory response. We therefore

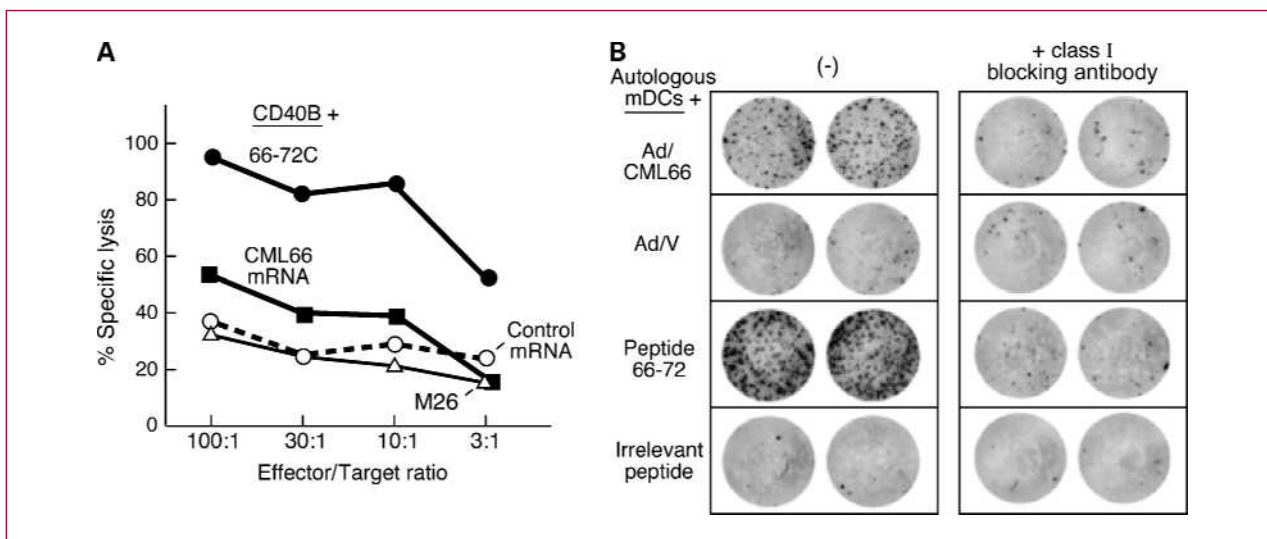
stimulated T cells isolated from Patient A, 5 years after DLI, against autologous DCs that were adenovirally transduced to express whole CML66 antigen, because this led to efficient expression of this protein within DCs (Supplementary Fig. S1A).

Ten days after this single stimulation against CML66-expressing DCs, CD8+ T cells from Patient A were tested by ELISpot for reactivity against a panel of overlapping peptides, encompassing the entire coding region of CML66. A total of 91 15-mers to 18-mers, which overlapped by 11 residues, were divided into 18 pools (Supplementary Table S2). Patient A was known to express HLA-A2+ and was CMV seropositive. As expected, Patient A CD8+ T cells stimulated with DCs expressing either the empty adenoviral vector or CML66 both elicited equivalent frequencies of CMV-specific reactivity against the known HLA-A2+–restricted PP65 peptide epitope (Supplementary Fig. S1B). Of the CML66-derived peptide pools, pool 15 elicited the greatest T-cell reactivity (80 spots per  $1 \times 10^5$  cells) by ELISpot, with 2.6-fold greater reactivity from CML66-stimulated cells compared with T cells stimulated against control DCs. Further testing of the six peptides contained within pool 15 revealed that the CD8+ T-cell reactivity was directed against the 18-mer 66-72 (CML66 residues 449-466, PKEMPCFLRHDV-DALLW; Supplementary Fig. S2).

**Peptide 66-72 contains a naturally processed, class I-restricted epitope of CML66.** We sought to determine whether peptide 66-72 reactive CD8+ T cells could recognize an epitope derived from processed whole antigen. As APCs, we used CD40L-expanded autologous B cells, which could efficiently express CML66 or irrelevant/control proteins following nucleofection with gene-specific mRNA (Supplementary Fig. S3). As shown in Fig. 1A, peptide 66-72-reactive T cells were cytolytic to autologous CD40L-expanded B cells expressing CML66 and APCs pulsed with peptide 66-72 but not against autologous CD40L-B cells expressing irrelevant antigen or pulsed with irrelevant peptide (i.e., the melanoma antigen MART1 or its peptide M26). These results show that, with forced expression, CML66 is processed to express an epitope containing peptide 66-72.

Peptide 66-72-reactive T cells were cloned by limiting dilution. Clones 3A1, 9A8, and 11A5 all specifically recognized autologous DCs pulsed with peptide 66-72 or expressing whole CML66 protein and were further expanded. Figure 1B shows that these interactions were class I restricted, because IFN- $\gamma$  secretion was blocked in the presence of the class I blocking antibody W6/32.

**Peptide 66-72C is the HLA\*B4403-restricted epitope of CML66.** To identify the minimal class I-binding epitope of CML66, 8-mer, 9-mer, and 10-mer peptides derived from the parental 18-mer, peptide 66-72, were pulsed onto autologous DCs and tested against clones 3A1, 9A8, and 11A5 by ELISpot. Figure 2 shows that maximal IFN- $\gamma$  secretion was observed by all three clones when tested against either the 8-mer HDVDALLW (designated as peptide 66-72C) or the 9-mer RHDVDALLW. The COOH terminal residue W seemed to be critical for the TCR-MHC



**Fig. 1.** Identification of a CML66-derived peptide epitope eliciting a T-cell response in DLI responder Patient A. A, peptide 66-72 contains a naturally processed epitope of CML66. By cytotoxicity assay, peptide 66-72-reactive T cells are cytolytic to cells expressing CML66 whole antigen (using autologous CD40L activated B cells, nucleofected with CML66 RNA as targets, black squares) or pulsed with peptide 66-72C (black circles) compared with CD40L-B cells nucleofected to expressing irrelevant antigen (MART1, open circles) or pulsed with irrelevant peptide (M26, open triangles). Representative results from three independent experiments. B, recognition of the peptide 66-72 epitope is HLA class I restricted. Peptide 66-72-specific T-cell clones (500 cells per well) were tested by ELISpot assay against autologous mature DCs either transduced to express whole CML66 antigen (Ad/CML66) or vector (Ad/V) or pulsed with peptide 66-72 or irrelevant peptide in the presence or absence of class I blocking antibody (W6/32 antibody).

complex interaction, because its absence resulted in loss of IFN- $\gamma$  secretion.

Patient A was known to express HLA A\*0203, A\*3301, B\*1301, B\*4403, Cw\*0304, and Cw\*0702. Comparison with the binding motifs from the SYFPEITHI database (20) showed that B\*4403 is the only HLA allele from Patient A's haplotype that could be predicted to accommodate W at the peptide COOH terminus (motif x[EMILDV]xxxxx[YFW]). The peptide binding scores were calculated using the NetMHCpan prediction system (21). The predicted score for the 8-mer peptide HDVDALLW and B\*4403 was 469.49 (weak binding), whereas all other combinations of peptides were predicted as nonbinding interactions. Therefore, the 8-mer HDVDALLW was considered the best candidate epitope and B\*4403 as restriction element.

We verified this prediction by testing the peptide 66-72-reactive T-cell clones against a series of EBV-immortalized B-cell lines that were pulsed with peptide 66-72 derived from individuals who shared at least one HLA allele with Patient A. Of the seven lines tested, four of four express HLA-B44-elicited reactivity from the CML66-reactive clones (Supplementary Fig. S4). These results were further confirmed in experiments using 721.22 cells, which in parental form are MHC class I deficient but were engineered to express only a single MHC class I allele (16). As shown in Fig. 2B, the CML66-reactive T-cell clones were all reactive against HLA-B\*4403-expressing cells but not cells expressing HLA-A\*0203, HLA-A\*3301, or HLA-B\*4402.

To confirm that peptide 66-72C is endogenously processed, we tested the CML66-specific T-cell clones against

the HLA-B4403-expressing human CD34+ acute myeloid leukemia cell line MUTZ-3 (22). MUTZ-3 cells express high levels of CML66 (Fig. 2C). By ELISpot, CML66-reactive T-cell clones recognized MUTZ3, and this recognition was abrogated in the presence of class I blocking antibody (Fig. 2D).

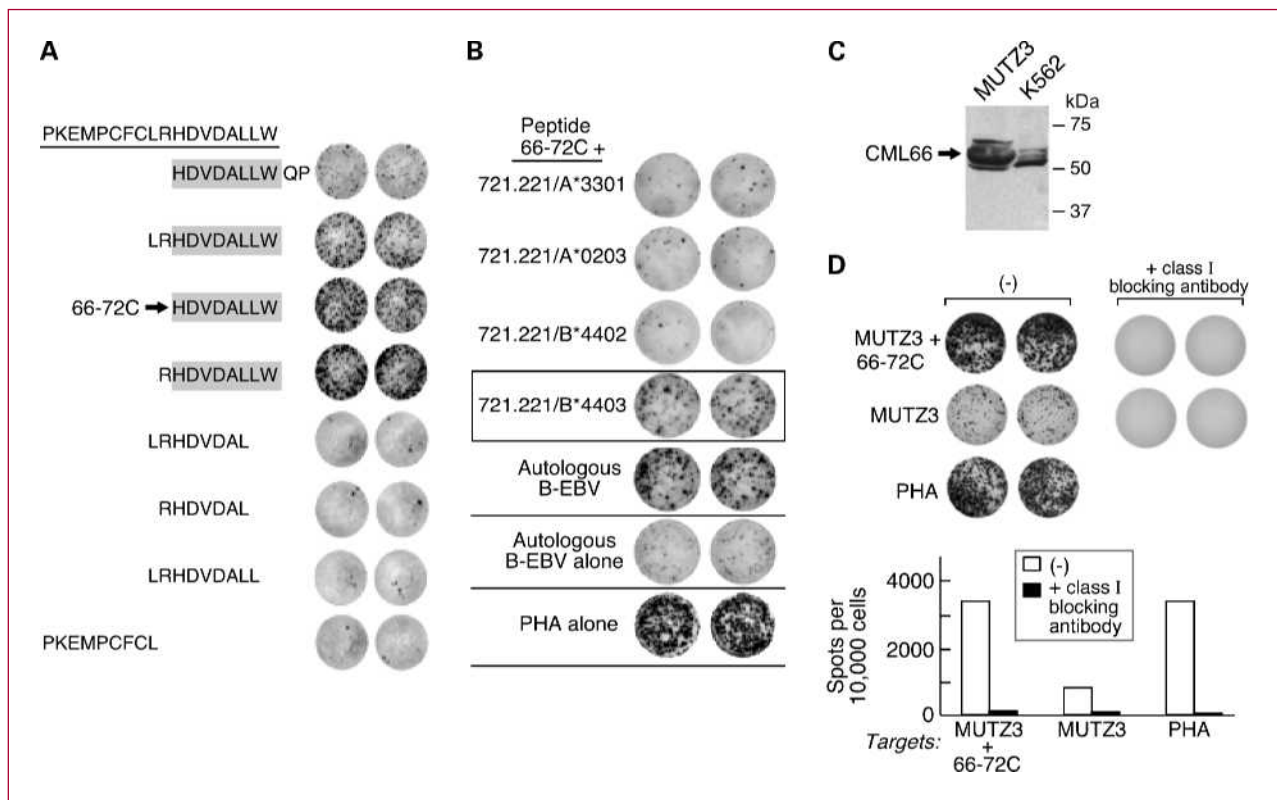
**CML66-specific CD8+ T-cell responses in peripheral blood and marrow are coordinated with B-cell responses following DLI.** We previously showed that CML66 elicits high-titer antibody reactivity in Patient A. To define the CML66-associated antibody epitope, we tested patient plasma against an array of 36 pools of overlapping CML66-derived 18-mers (see Supplementary Table S2). Each peptide was represented in two separate pools. Post-DLI plasma, diluted 1:200, was reactive on ELISA assay against two pools—one containing peptides 66-31 through 66-35 and the other containing peptides 66-28, 66-33, 66-38, 66-43, and 66-48 (Fig. 3A). Hence, we concluded that the CML66 B-cell epitope was contained within peptide 66-33 (GFYVSLEWVTISKKNQDNK, residues 198-216). Plasma reactivity to peptide 66-33 was also detected at dilutions 1:500 and 1:1,000. Although we cannot exclude that the patient plasma also contained antibodies reactive to nonlinear epitopes from CML66, we found the pattern of reactivity against 66-33 consistent with our previous results using recombinant whole protein as coating ELISA antigen (13). We detected clear reactivity against peptide 66-33 developing 2 to 3 months, which increased by 12 months following DLI when the patient developed molecular remission (Fig. 4) not present before DLI. The B-cell and T-cell epitopes of the CML66

antigen are thus positioned at different regions of CML66 (Fig. 3B).

We next tested serial PBMC and marrow samples for evidence of *in vivo* T-cell reactivity against peptide 66-72C. PBMC and marrow before and in the months following DLI were thawed and presensitized to CML66 once using autologous DCs expressing CML66. Ten days later, CD8+ T cells were isolated and tested by ELISpot against peptide 66-72C-pulsed autologous DCs. In peripheral blood, antigen-specific CD8+ T-cell reactivity was absent before DLI but then readily detectable between 1 and 3 months following DLI (~45 IFN- $\gamma$ -secreting CD8+ T cells/50,000 cells) and thereafter waned (Fig. 4A). More dramatically, marrow CML66-specific T-cell reactivity peaked at ~1 month following DLI, coinciding with maximal peripheral blood response but was of >10-fold greater frequency. We observed that marrow-derived and blood-derived CML66-specific T-cell responses peaked in the few weeks preceding cytogenetic elimination of BCR-ABL expressing cells within the marrow.

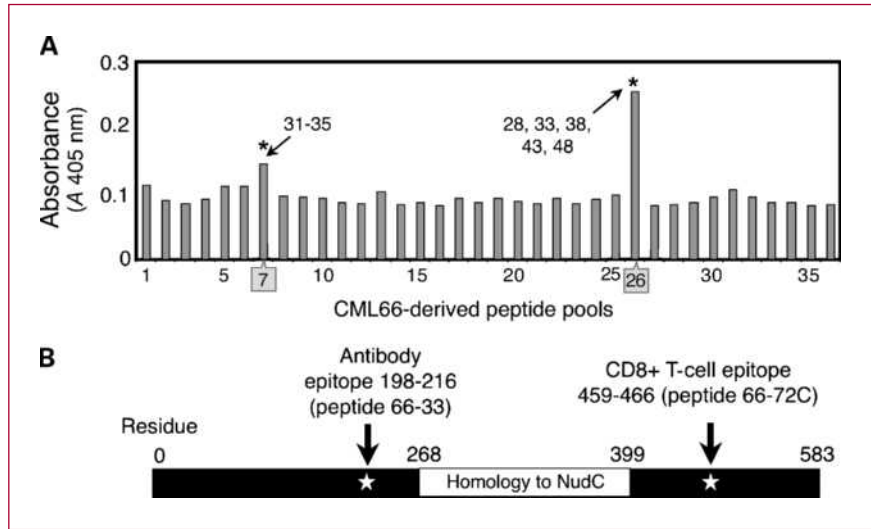
**Donor-derived CML66-specific T-cell clones are long lived and present in marrow before DLI.** Intriguingly, ELISpot

analysis revealed that CML66-reactive T cells were present in marrow before DLI although at 5-fold less levels than the height of the DLI-associated response (100 spots per  $5 \times 10^4$  cells). Chimerism analysis of pre-DLI marrow-derived T cells revealed that they were fully donor derived (Fig. 4B). To detect the CML66 epitope-specific T-cell clones *in vivo*, we developed a sensitive nested PCR assay to amplify CDR3-specific sequences unique to the TCRs of the clones. As summarized in Fig. 5, we first identified the specific CDR3 sequences of clones 3A1, 9A8, and 11A5 by reverse transcription-PCR using primers specific for each of the 24 V $\beta$  subfamilies, which were pooled into five mixtures. We had insufficient material to complete the analysis for clone 3A1. However, as shown in Fig. 5A, clone 11A5 expressed a V $\beta$  subfamily included in pool 3, which was identified as V $\beta$ 10 (Fig. 5B), whereas clone 9A8 expressed V $\beta$ 6 (data not shown). By spectratyping, the amplified V $\beta$ 6 and V $\beta$ 10 bands were confirmed to be derived from clonal cells because only single peaks—representing CDR3 sequences derived from a single rearrangement—were observed (Fig. 5C). Next, CDR3 region-specific quantitative PCR primers were designed such that the



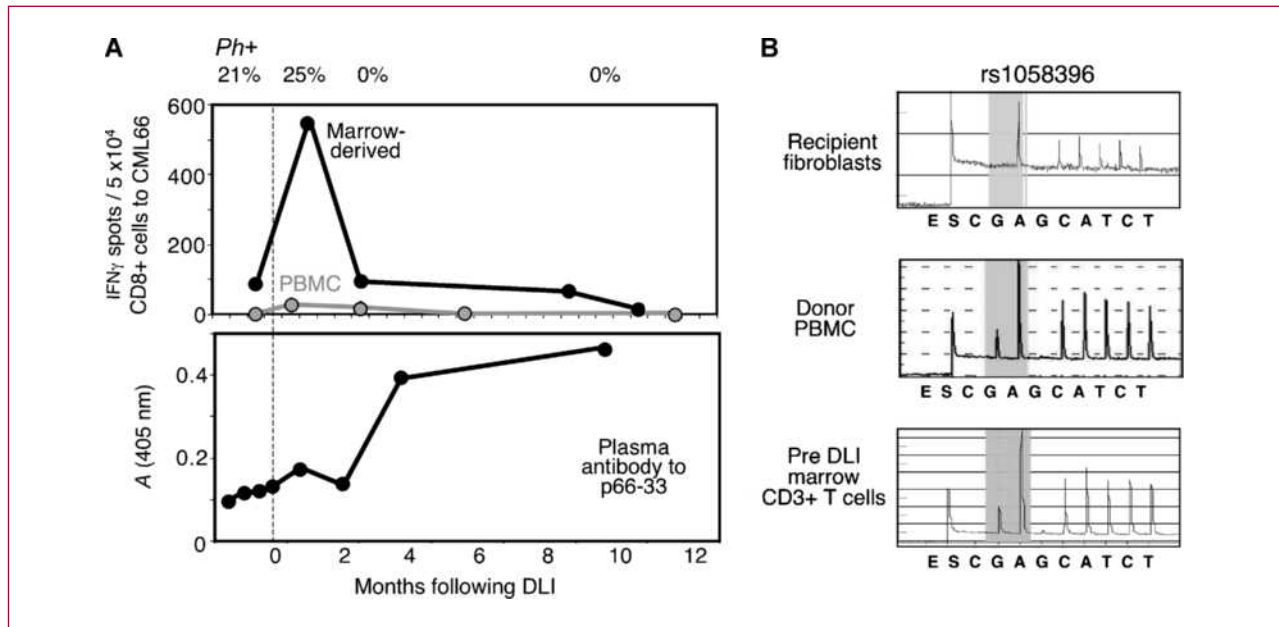
**Fig. 2.** CML66 is processed and presented by CD34+ CML66+ myeloid leukemia cells in the context of HLA B4403. A, the minimal epitope of the 18-mer parental peptide 66-72 (PKEMPCFCLRHDVDALLW) is an 8-mer peptide (indicated by the arrow, designated peptide 66-72C). By ELISpot, 5,000 antigen-specific T cells were tested per well against autologous matured DCs pulsed with various 8-mer, 9-mer, and 10-mer peptides derived from peptide 66-72. Reactivity was detected among the peptides containing the 8-mer HDVDALLW (shaded). B, recognition of peptide 66-72C is restricted to HLA-B\*4403 (boxed). Peptide 66-72C-pulsed 721.221 cells (50,000 cells per well) expressing the individual HLA A and B alleles of Patient A were tested against 1,000 cells of clone 9A8. Similar results were observed with clones 3A1 and 11A5. C, protein lysates generated from MUTZ3 and K562 cells (20  $\mu$ g/lane) were immunoblotted against the CML66 monoclonal antibody 22F. D, the 66-72-specific T-cell clone recognizes MUTZ3 cells on ELISpot assay. This interaction can be blocked in the presence of class I MHC blocking antibody. These results were observed for clones 3A1, 9A8, and 11A5.

**Fig. 3.** The CML66 B-cell and T-cell epitopes are positioned in different regions of the antigen. A, post-DLI plasma of Patient A is reactive against peptide pools 7 and 26, which contain peptide 66-33 (\*). Plasma (1:200 dilution) was tested on ELISA in duplicate against an array of peptide pools, each containing five to six peptides per pool (pools described in Supplementary Table S2). B, schematic representation of the positions of the B-cell and T-cell epitopes of CML66.

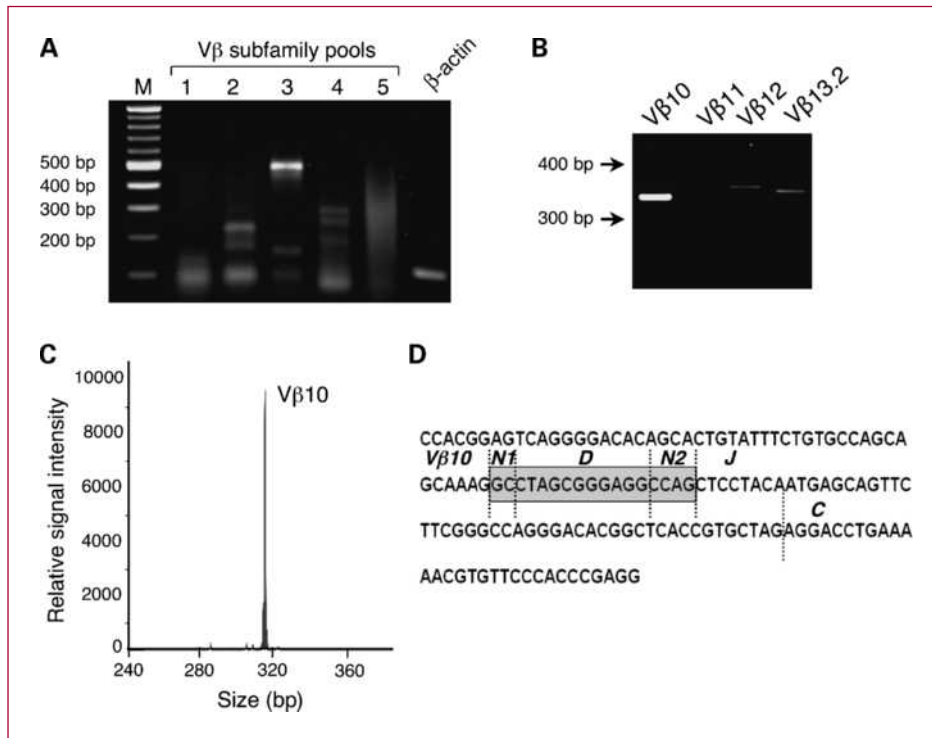


probe sequences were positioned within the region of junctional diversity (Fig. 5D).  
 The results of our analysis are depicted in Fig. 6. As expected, our assays efficiently amplified RNA derived from the 9A8 and 11A5 clones, but not from irrelevant T-cell clones, normal marrow, nor normal PBMC, including T cells from the transplant donor (Supplementary

Table S4). Clone 11A5, however, was detectable by nested PCR in RNA directly extracted from Patient A's peripheral blood only following DLI, whereas this same clone was detectable in marrow prior and again following DLI, with detectability tapering by 12 months post-DLI. As shown in Fig. 6, clone 11A5 was detectable in marrow for several months before the patient received DLI treatment, when



**Fig. 4.** Expansion of the CML66-specific T-cell population following DLI, which is coordinated with the antigen-specific B-cell response. A, expansion of CML66-reactive T cells from peripheral blood and marrow 1 to 3 mo following DLI, detected by IFN- $\gamma$  ELISpot following presensitization with autologous DCs transduced to express CML66 and then restimulation with peptide 66-72C-pulsed DCs. T-cell reactivity was measured by calculating the number of spots in duplicate wells against CML66-expressing targets over control target cells. Plasma reactivity (diluted 1:200) before and after DLI against peptide 66-33 was measured by ELISA assay. Peptide-specific absorbance was calculated as the difference in duplicate wells between absorbance (peptide) and absorbance (reagent control). Development of immune responses is depicted in relationship to percentage of BCR-ABL (or, Philadelphia chromosome positive [Ph+]) cells in the marrow. B, marrow-infiltrating T cells before DLI are donor derived. Genomic DNA was extracted from recipient fibroblasts, donor PBMC, and pre-DLI marrow CD3+ cells and amplified for the region of polymorphism around single-nucleotide polymorphism rs 1058396. Pyrosequencing revealed the amplicon sequence of marrow-infiltrating T cells to be identical to the donor.



**Fig. 5.** Design of T-cell clone-specific primers. A, RNA from the CML66 T-cell clone 11A5 expressed a Vβ subfamily included in primer pool 3. All Vβ subfamilies generated amplicons between 300 and 400 bp in length when using the Cβ reverse internal primer. B, of the primers contained in primer pool 3, clone 11A5 was found to express Vβ10. C, the Vβ10 amplicon generated a monoclonal peak by spectratyping. D, clone-specific primers for 11A5 were designed based on the sequence of the clone-specific CDR3 rearrangement, such that the quantitative PCR probe was positioned in the region of junctional diversity (shaded region).

disease relapse was apparent by marrow cytogenetic evaluation. Five years after DLI, this clone was still present at very low frequency, as it could only be detected following antigen-specific stimulation (data not shown). In contrast, clone 9A8 showed a very different kinetic: although it was not detectable in blood nor marrow before DLI nor within the first 24 months after DLI, it could be detected by nested PCR directly from PBMC and marrow 4 to 5 years after DLI. Together, these results show that CML66 elicits long-lived cellular immunity.

## Discussion

The role of adaptive B-cell immunity in GvL responses following allogeneic HSCT has not been well characterized. We previously uncovered high-titer antibodies, developing in the setting of DLI-induced GvL responses, that targeted a broad array of intracellular, overexpressed, and nonpolymorphic leukemia-associated antigens. Although our studies are admittedly restricted to the analysis of responses against a single antigen in a single patient, our data provide intriguing evidence that leukemic antigen-specific antibodies can mark functional cytolytic CD8+ responses after HSCT. We did detailed studies to dissect the kinetics of B-cell and T-cell immunity developing against a response-associated antigen, CML66, in an individual with long-lasting remission in the absence of graft-versus-host disease. We delineate a temporal sequence of activity by immune effectors in this patient, in which CML66-specific CD8+ T-cell responses are first detectable in the marrow before DLI. Following infusion

of donor CD4+ cells, we then observed antigen-specific CD8+ T-cell reactivity expanding in both blood and marrow together with appearance of high-titer B-cell responses directed against the same antigen coincident with attaining clinical remission. These data provide the first description of coordinated adaptive immunity developing against a bona fide leukemia-associated antigen in marrow and blood following transplantation.

We previously showed that patients with robust GvL responses to DLI therapy develop potent humoral immunity whereas nonresponders do not. Other investigators have shown the potential of posttransplant B-cell responses to provide functional antitumor immune effects.<sup>5</sup> We discovered that T-cell responses immediately preceded cytogenetic and increased B-cell responses, and these results suggest T cell-mediated lysis of leukemia cells increases the accessibility of overexpressed antigens to B cells for antibody production. Given the long-lasting immunity associated with DLI, a focus of ongoing studies is determining whether antibodies also serve to enhance and amplify GvL to increase cross-presentation of antigen to APCs through antibody-mediated uptake of intracellular antigens (23–25).

GvL effects have been commonly attributed to cellular targeting of alloantigens with lymphohematopoietic restricted expression (26), whereas the extent and identity

<sup>5</sup> M.A. Biernacki, O. Marina, F.L. Liu, et al. Antigen targets of remission-inducing immune therapy are expressed on CML progenitor cells (in revision) 2009.



of GvL targets that are leukemia associated, such as CML66, are unknown. Antigens with leukemia-restricted expression include products of chromosomal translocations and mutated, overexpressed, or aberrantly expressed self-proteins (27). For CML, these include the gene product of BCR-ABL, WT-1, and proteinase 3, although the ability of these to elicit functional T-cell responses following allotransplant has been unclear (28, 29). Our current studies uniquely provide evidence that GvL responses are comprised at least partly of donor immunity directed against nonpolymorphic leukemia-associated antigens. Furthermore, serologic responses may represent an efficient approach to identify response-associated leukemia-associated antigens. In addition to CML66, we have previously identified numerous B cell-defined antigens targeted by GvL in patients who have undergone allotransplant or DLI (10).<sup>5</sup> These were found to be highly expressed in leukemic cells, were without sequence polymorphisms between donor and recipient, and hence are not alloantigens. Although we have not systematically examined concurrent T-cell responses to these antigens, our current results suggest that a subset will likely elicit T-cell mediated tumor-specific recognition. Consistent with these findings, Nishida et al. recently identified several T-cell clones, reactive against tumor and not to recipient hematopoietic cells, developing in patients with clinical response following nonmyeloablative transplant for chronic lymphocytic leukemia (9). Together, a major implication of our data is that leukemia-associated antigens discovered in the context of therapeutic response to allotransplant may be more numerous than previously appreciated. Their discovery may enable the development of strategies to more rationally enhance GvL effects without inciting graft-versus-host disease or even antitumor immunity in the absence of allografting.

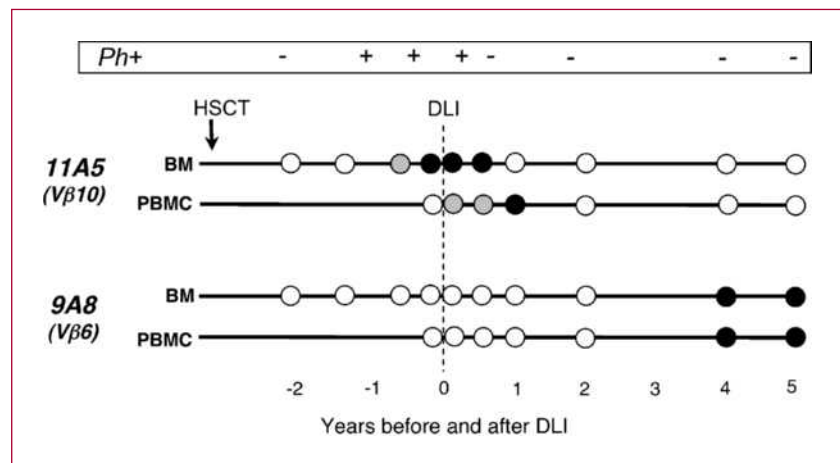
Our analysis of individual CML66-specific CD8+ T-cell clones reveal several important insights regarding GvL. Firstly, as an antigen, CML66 seems capable of eliciting polyclonal T-cell reactivity, as we discovered different V $\beta$  specificities among the analyzed clones. Although we pre-

dominantly focused on two clones here, our original limiting dilution analysis yielded far more antigen-specific clones, and probably several other V $\beta$  specificities could have been uncovered. Moreover, CML66 likely contains other immunogenic regions (i.e., within pools 13 and 17; Supplementary Fig. S1B) and epitopes within the context of other MHC class I-restricting alleles. Suemori et al., for example, recently described a HLA-A24-defined epitope (residues 76-84), which elicits recognition of whole CML66-expressing leukemia cells (30, 31).

Secondly, CML66-specific clones are remarkably enduring *in vivo*. Even 5 years after the patient's clinical response, we could still identify the 11A5 TCR that was present before DLI. Moreover, new CML66-specific T cells were recruited over time, as the 9A8 clone seemed only years after DLI. DLI results in T-cell neogenesis and reconstitution of T-cell repertoire diversity (32–34). Intermittent molecular detection of BCR-ABL transplant has been reported in successfully transplanted CML patients, and continued immune surveillance is likely critical in maintaining durable remission (35). Thus, our data suggest that one facet of DLI-induced GvL responses is the generation of enduring leukemia antigen-specific cellular immunity.

Thirdly, our studies provide supportive evidence that leukemia cells actively suppress the function of donor CD8+ cytolytic T cells. We could identify CML66-specific T cells residing in marrow before DLI, but we only observed functional T-cell responses resulting in high IFN- $\gamma$  secretion after DLI. Marrow has been long appreciated as a reservoir for high-avidity memory B and T cells that are critical for sustaining long-term humoral and cellular immunity (36–38). Marrow is also a major priming site for T-cell responses to blood-borne antigens (39). Recently, model systems have underscored the essential role of central memory effector T cells for sustaining long-lasting immunity (40), and the marrow compartment has been shown to harbor antigen-specific central memory T cells to tumor-associated antigens (8, 41–43). At the same time, marrow is a known site of residual disease in hematologic malignancies. Studies in viral immunity have

**Fig. 6.** Pacing of accumulation of CML66-specific T cells before and after DLI in peripheral blood and marrow of Patient A, detected by clone-specific CDR3 nested PCR. Each circle represents a tested sample. White circle, negative/no amplification; gray circle, positive/amplification detected; black circle, double positive/amplification detected and amplification level is more than median level of all positive samples. Also shown is monitoring of tumor burden based on detection of Ph+ (BCR-ABL) chromosomes by marrow cytogenetic evaluation.



described T-cell “exhaustion” in the setting of chronic antigen exposure (44). Leukemia cells that are residing in the marrow are a source of persistent antigen and likely exert a myriad of inhibitory effects of immunity (28).

In closing, our studies suggest a new paradigm for understanding GvL, which involves immunity against leukemia-associated antigens together with alloantigens and which elicit complex immunity. Evidence of tumor-specific immunity following HSCT has several implications for developing approaches to stimulate immunity against malignancy in even the autologous setting. For example, generating co-ordinated B-cell and T-cell immunity may be an important characteristic to mimic. Secondly, our studies underscore the importance of efficient antigen priming to expand the pool of T cells with desired specificity whose function can be secondarily modulated. Many more questions are raised by our studies, including defining the relationship between antigen-specific CD4+ T cells against CML66 with developing CD8+ and antibody responses and detailing how B-cell immunity functions to modulate T-cell responses. Future studies with this GvL antigen will likely provide further mechanistic insights that can be applied to developing effective vaccination strategies.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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