

## Chronic Lymphocytic Leukemia Monitoring with a Lamprey Idiotope-Specific Antibody

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### Abstract

For antigen recognition, lampreys use leucine-rich repeats (LRR) instead of immunoglobulin V-(D)-J domains to generate variable lymphocyte receptors (VLR) of three types: VLRA, VLRB, and VLRC. VLRB-bearing lymphocytes respond to immunization with proliferation and differentiation into plasmacytes that secrete multivalent VLRB antibodies. Here, we immunized lampreys with B cells from patients with chronic lymphocytic leukemia (CLL) to generate recombinant monoclonal VLRB antibodies, one of which, VLR39, was specific for the donor CLL cells. The target epitope of VLR39 was shown to be the complementarity determining region 3 (CDR3) of the heavy chain variable region (VH) of the B-cell receptor. Using this antibody to monitor the CLL donor after chemoimmunotherapy-induced remission, we detected VLR39<sup>+</sup> B cells in the patient 51 months later, before significant increase in lymphocyte count or CD5<sup>+</sup> B cells. This indication of reemergence of the leukemic clone was verified by VH sequencing. Lamprey antibodies can exhibit exquisite specificity for a protein epitope, a CLL signature VH CDR3 sequence in this case, and offer a rapid strategy for generating anti-idiotype antibodies for early detection of leukemia recurrence. *Cancer Immunol Res*; 1(4); 223–8. ©2013 AACR.

### Introduction

The adaptive immune system of the extant jawless vertebrates, lamprey and hagfish, offers an alternative to monoclonal antibody (mAb) technology as a method to produce specific binding molecules. Instead of immunoglobulin (Ig)-based antigen receptors, lampreys and hagfish use variable lymphocyte receptors (VLR) composed of leucine-rich repeat (LRR) modules for antigen binding (1, 2). Similar to the Toll-like receptors and many other LRR proteins, the VLR proteins form a curved solenoid structure (3). Antigen binding occurs on the concave surface formed by the parallel  $\beta$ -strands of the "LxxLxLx" motifs in the variable LRR subunits; an important additional contact is contributed by an extended loop of highly variable sequence in the C-terminal LRR (4–8).

Lampreys have three lymphocyte lineages, each of which expresses a different VLR type. The VLRA<sup>+</sup> and VLRC<sup>+</sup> lym-

phocytes are T-like cells that exclusively express their VLRs as cell surface molecules (9, 10), whereas VLRB<sup>+</sup> lymphocytes resemble mammalian B cells in that they express their antigen receptors on the cell surface, respond to immunization with proliferation, and differentiate into plasma cells that secrete disulfide-linked multimeric VLRB antibodies (4, 11). The *VLRB* genes are somatically assembled in lamprey B-like cells by a gene conversion-like process in which sequences from flanking *LRR* gene cassettes are randomly and sequentially incorporated in a piece-wise fashion into an incomplete germline *VLRB* gene to generate a potential repertoire of more than 10<sup>15</sup> unique *VLRB* genes (1, 2, 12, 13). Allelic exclusion ensures that each individual lymphocyte assembles and expresses a unique *VLRB* gene (1, 13, 14). Recombinant monoclonal VLRB antibodies can be produced by making a *VLRB* cDNA library from immunized lampreys, expressing the derivative *VLRB* clones in a secretory cell line and selecting VLRB antibodies based on antigen specificity and affinity (4, 7, 15, 16). The single-chain polypeptide nature of the VLR protein should make them more amenable for molecular engineering compared with Ig-based antibodies, which require the assembly of complementary heavy and light chains (4, 17).

To determine the feasibility of producing lamprey VLRB antibodies with tumor cell specificity, lampreys were immunized with cells from a patient with B-cell chronic lymphocytic leukemia (CLL), and derivative VLRB clones were screened for CLL specificity. Among the lamprey VLRB antibodies produced against human mononuclear blood cells (MNC), we identified a monoclonal VLRB antibody, VLR39, which preferentially recognized the donor CLL clone. Here, we describe the B-cell receptor (BCR) idiotope specificity of VLR39 and the potential

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**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

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doi: 10.1158/2326-6066.CIR-13-0052

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use of this anti-idiotypic antibody as a monitoring reagent for early detection of CLL recurrence.

## Materials and Methods

### Cells and cell lines

Blood samples were obtained with informed consent from patients with CLL and healthy adults in studies approved by the Institutional Review Boards of Emory University (Atlanta, GA), the University of Alabama at Birmingham (UAB; Birmingham, AL), and the North Shore-LIJ Health System (Manhasset, NY), in accordance with the Declaration of Helsinki. MNCs isolated from whole blood by density gradient centrifugation using Lymphocyte Separation Media (Mediatech) were examined immediately or cryopreserved at  $-150^{\circ}\text{C}$  in FBS supplemented with 10% dimethyl sulfoxide. HEK-293T cells (generously provided by Dr. Tim Townes, UAB) were maintained in Dulbecco's Modified Eagle Medium supplemented with 5% FBS at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . B-cell lines were maintained in RPMI-1640 media supplemented with 10% FBS at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . The EBV-transformed B cells were kind gifts from Dr. Lou Justement (UAB). The B-cell phenotypes of the 697, Daudi, Ramos, and SU-DHL-6 cells were verified by flow cytometry.

### Antibodies and flow cytometry

The 4C4 mouse IgG2b/ $\kappa$  mAb against the invariant VLRB stalk region was described previously (11). R-phycoerythrin (RPE)-conjugated goat anti-mouse IgG polyclonal antibodies (Southern Biotech) were used for detection of 4C4. The 8A5 VLRB-specific mouse IgG1/ $\kappa$  mAb was generated by immunization with full-length VLRB protein, labeled with Alexa Fluor 488 Protein Labeling Kit (Invitrogen), and recognizes 80% of VLRB clones. Mouse anti-human antibodies CD5-FITC, CD5-APC, CD19-PE, propidium iodide, and 7-AAD were from BD Biosciences, and CD19-PECy7 was from Southern Biotech.

### Animal maintenance and immunization

Two lamprey larvae, maintained as described earlier (2), received intracoelomic injections of  $1 \times 10^7$  blood MNCs from a newly diagnosed patient with CLL on days 0, 14, and 28, before sacrifice on day 42 for collection of buffy coat and plasma (2). All experiments were approved by the Institutional Animal Care and Use Committee at UAB.

### VLRB cDNA library construction, recombinant VLRB expression, and screening for CLL reactivity

Buffy coat leukocytes from an immunized lamprey with the highest titer of donor CLL-reactive VLRB antibodies were used to construct a VLRB cDNA library; individual clones were transfected into HEK-293T cells to obtain recombinant VLRB antibodies (4). To assess the antigen specificity of the monoclonal VLRB antibodies, MNCs from patients with CLL and healthy adults were sequentially incubated with VLRB transfectant supernatants, followed by 4C4 anti-VLRB mAb and then RPE-conjugated goat anti-mouse IgG polyclonal antibodies, before blocking with 5% mouse serum and staining for CD5 and CD19. Cells were washed in PBS between incubation

steps, and propidium iodide was added before the cells were analyzed by flow cytometry.

### Purification of monoclonal VLR39 antibody

Purified VLRB proteins were obtained by PCR cloning of the VLR39 cDNA into a pIRES-puro-2 vector (Clontech) modified to introduce a hexahistidine-tag between the lamprey VLRB signal peptide and LRRNT before transfection into HEK-293T cell lines. Stable transfectants were selected with  $2 \mu\text{g}/\text{mL}$  puromycin (Sigma), and recombinant VLRB protein was purified from supernatant via the hexahistidine tag using Ni-NTA agarose (Qiagen). Briefly, 1/10 volume of a  $\times 10$  binding buffer (500 mmol/L  $\text{NaH}_2\text{PO}_4$ , pH 8.0, 1.5 mol/L NaCl, 100 mmol/L imidazole) was added to the transfectant supernatant before passage over the Ni-NTA agarose column, which was washed with 5 column volumes of wash buffer (50 mmol/L  $\text{NaH}_2\text{PO}_4$ , pH 8.0, 300 mmol/L NaCl, 20 mmol/L imidazole, 0.05% Tween-20) before elution with 50 mmol/L  $\text{NaH}_2\text{PO}_4$ , pH 8.0, 300 mmol/L NaCl, 250 mmol/L imidazole, and 0.05% Tween-20. The eluate was concentrated by centrifugation using an Amicon Ultra-15 30K Centrifugal Filter Device (Millipore).

### Cloning, expression, and sequence analysis of CLL donor BCR

RNA from the CLL donor MNCs was converted into cDNA and expressed Ig heavy-chain and light-chain variable (VH/VL) regions were sequenced and analyzed as described previously (18, 19). The amplified VH and VL regions from the CLL donor were cloned and expressed as recombinant IgG1 antibodies (20), then papain-treated to yield Fab fragments for use in ELISA analysis of VLR39 binding. Reverse-transcription PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as described previously (20, 21). A single-chain variable fragment (scFv) of the CLL donor BCR was constructed by amplifying the VH and VL regions from the recombinant CLL donor IgG1 mAb expression plasmid using overlap-extension PCR with KOD Hot Start DNA Polymerase (Novagen) following a published protocol (22). Primer and PCR cycle profile are detailed in Supplementary Materials and Methods. The CLL donor scFv plasmid was transfected into HEK-293T cells using polyethylenimine as described earlier (4). Transfected cells were dissociated from culture plates using Cellstripper (Mediatech) and harvested 48 to 72 hours after transfection. Cell surface expression of CLL donor scFv was verified by flow cytometric analysis for myc-tag expression using Anti-Myc Tag, clone 4A6, Alexa Fluor 488 conjugate (Millipore). Primer and PCR cycle profiles for constructing the two scFv mutants are described in Supplementary Materials and Methods.

### Analysis of VLR39 specificity

For ELISA, 96-well EIA/RIA Plates (Corning) were coated overnight at  $4^{\circ}\text{C}$  with  $5 \mu\text{g}/\text{mL}$  of recombinant Fab fragments either from the donor CLL or from another CLL with unmatched V genes. Nonspecific binding was blocked with TBS-T/1% milk before incubation with between 1:20 and 1:20480 PBS dilution of 0.5 mg/mL purified VLR39 protein, which was detected with 4C4 anti-VLRB mAb followed by

alkaline phosphatase-conjugated goat anti-mouse IgG polyclonal antibodies (Southern Biotech). For flow cytometric analysis, MNCs from patients with CLL and healthy adults were sequentially incubated with 0.04 mg/mL purified VLR39 protein, followed by Alexa Fluor 488-conjugated 8A5 anti-VLRB mAb, and then CD5, CD19, and 7-AAD staining; cells were washed between each incubation step.

## Results and Discussion

### Identification of a donor CLL-specific monoclonal VLRB antibody

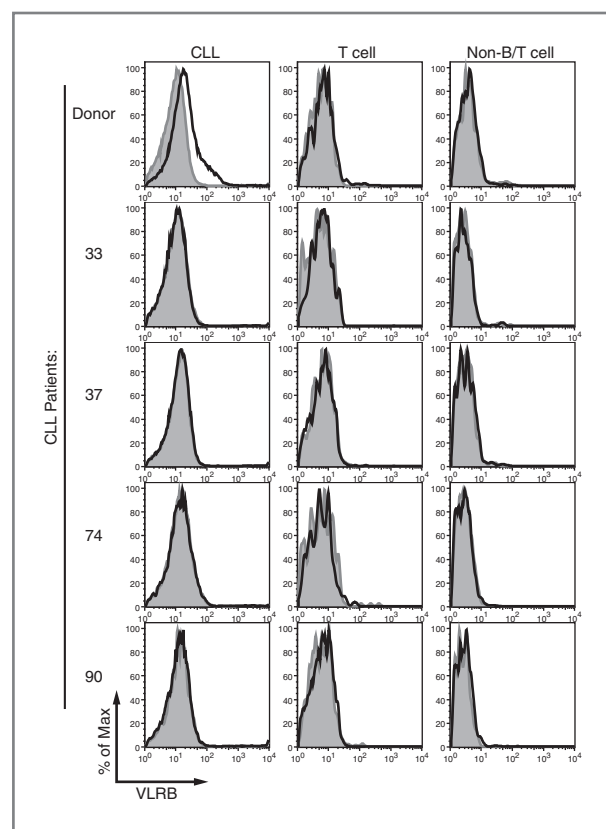
Individual clones selected randomly from the CLL-immunized *VLRB* cDNA library were transfected into HEK-293T cells, and the transfectant supernatants were assessed by Western blot analysis for the presence of secreted VLRB antibodies. Fourteen of 36 transfectants secreted VLRB that was detectable by Western blot analysis, and these supernatants were analyzed by immunofluorescence for binding to viable donor CLL cells. One monoclonal VLRB antibody, VLR39, reacted with the donor CLL cells and not those from other patients with CLL (Fig. 1), lymphocytes, or other types of blood cells

from healthy adults or with B-cell leukemia/lymphoma lines (Supplementary Fig. S1). The low mean fluorescence intensity of the VLR39 staining for the donor CLL could be due to the relatively inefficient expression and secretion of VLR into the supernatant by transfected HEK-293T cells. To address this issue, the VLR39 supernatant was pooled and the purified protein was used for subsequent analyses. Staining with purified VLR39 protein, rather than with supernatant from transiently transfected HEK-293T cells, improved the staining intensity of the donor cells in subsequent experiments.

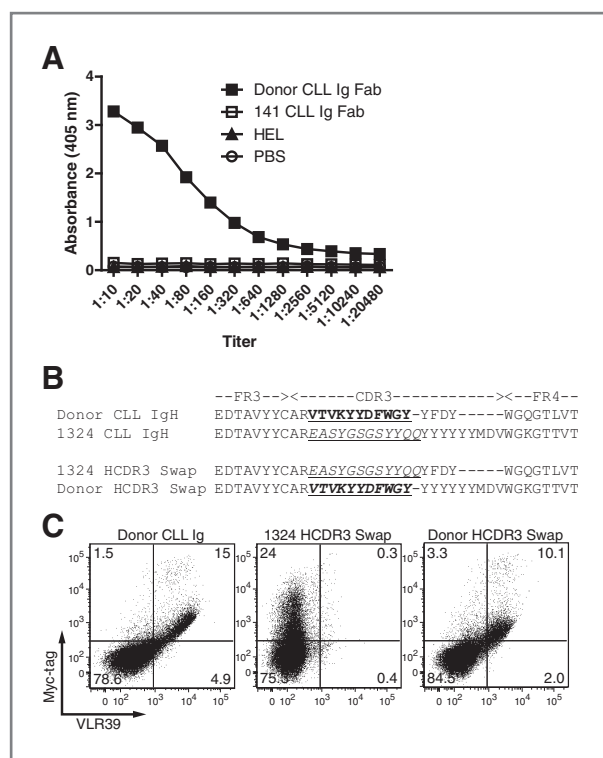
### VLR39 is specific for the VH CDR3 sequence of the donor CLL

To examine the possibility that VLR39 recognizes an Ig idiotype (Id) determinant on the donor CLL clone, the VH and VL regions of the leukemic clone from the donor CLL and those from another patient with CLL, 141, were sequenced and expressed as recombinant Fab fragments. The donor CLL *VH/VL* genes were composed of the *IGHV1-69\*01*, *IGHD3-3\*01*, *IGHJ4\*02*, and the *IGKV1-33\*01* and *IGKJ3\*01* alleles, whereas the control 141 CLL *VH/VL* genes were composed of the *IGHV4-34\*01*, *IGHJ5\*02*, *IGHD2-2\*01*, and *IGKV1-27\*01* and *IGKJ2\*01* alleles. When tested for reactivity by ELISA, VLR39 was found to react only with the donor CLL Ig Fab and not with the 141 CLL Ig Fab comprising different *VH/VL* gene sequences (Fig. 2A). Id specificity was tested against other CLL cells selected for partial overlap of their VH sequences with the donor CLL BCR sequence (Supplementary Table S1). VLR39 failed to react with tumor cells from 26 additional patients with CLL (Supplementary Fig. S2) even though the BCRs of 7 patients with CLL (1153, 1012, 1640, 1324, 1333, 352, 1397) shared identical VH CDR1 and CDR2 amino acid sequences with the donor CLL cells. Moreover, the BCRs of 3 other patients with CLL (1371, 336, 758, 1330) shared identical IGHJ sequences comprising the 3' end of the VH CDR3 (Supplementary Figure S3).

The exquisite Id-specificity of VLR39 was surprising, given that most Ig-based monoclonal anti-Id antibodies cross-react with serum Ig (23) and B cells (24) from healthy individuals. Of note, the CDR3 amino acid sequences for the 26 patients with CLL tested were all different from each other, suggesting that the VLR39 antibody recognizes the unique VH CDR3 sequence of the donor CLL. To test this interpretation, the VH/VL components of the CLL BCR from the donor and two mutants (1324 HCDR3 swap and donor HCDR3 swap) were expressed by transfected cells as an scFv with a myc-tag (Fig. 2B) and evaluated for anti-myc and VLR39 binding by immunofluorescence flow cytometry analysis (Fig. 2C). For the scFv mutant 1324 HCDR3 swap, the CDR3 signature sequence of the donor CLL VH was replaced with the CDR3 signature sequence of the 1324 CLL VH. For the other scFv mutant, donor HCDR3 swap, the CDR3 signature sequence of the 1324 CLL VH was replaced with the CDR3 signature sequence of the donor CLL VH. The 1324 HCDR3 swap transfectants failed to stain with VLR39, whereas the donor CLL Ig and donor HCDR3 swap transfectants costained with VLR39 and anti-myc, confirming that the donor CLL VH CDR3 region is the recognition site for the VLR39 antibody.



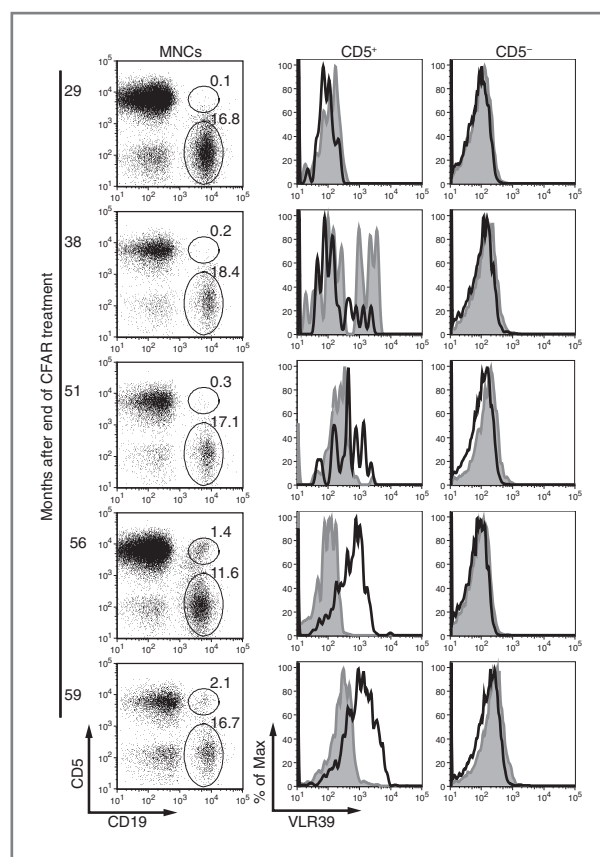
**Figure 1.** Flow cytometric analysis of monoclonal VLR39 reactivity. VLR39 binding (black line) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). Histogram of VLR39 binding to CLL donor (A) and 4 other CLL patient lymphocytes gated on CLL cells (CD5<sup>+</sup>/CD19<sup>+</sup>), T cells (CD5<sup>+</sup>/CD19<sup>-</sup>), and non-B/T cells (CD5<sup>-</sup>/CD19<sup>-</sup>).



**Figure 2.** ELISA and flow cytometric analysis of VLR39 binding to donor CLL Igs. **A**, comparison of VLR39 binding to recombinant Fab fragments containing the *VH/VL* genes of the CLL clone from the patient donor and from another patient with unmatched *VH/VL* genes. ELISA of VLR39 binding to donor CLL Ig Fab (black squares), 141 CLL Ig Fab (white square), hen egg lysozyme (HEL; black triangles), and PBS (white circles) coated wells. **B**, sequence alignment of HCDR3 region from donor CLL IgH, 1324 CLL IgH, and the two scFv mutants, 1324 HCDR3 swap and donor HCDR3 swap. The HCDR3 signature sequences of donor CLL (bold, underlined text) and the HCDR3 signature sequences of patient with CLL 1324 (italic, underlined text) were swapped with each other to construct the two scFv mutants. **C**, flow cytometric analysis of VLR39 binding to HEK-293T cells transfected with donor CLL Ig scFv or the two scFv mutants. Myctag was costained to measure scFv surface expression.

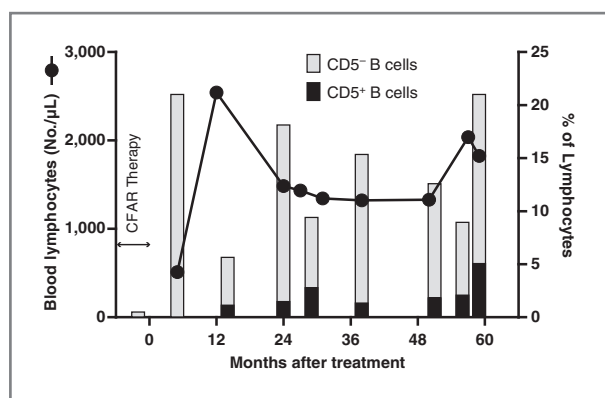
### VLR39 antibody monitoring for CLL recurrence after chemoimmunotherapy-induced remission

The exquisite specificity of VLR39 for the CLL signature HCDR3 epitope and the absence of native Fc-like-binding receptors for the lamprey antibody on human cells suggested that this lamprey antibody would be an ideal reagent for tracking the neoplastic clone in the CLL donor after treatment with multiple infusions of cyclophosphamide, fludarabine, alemtuzumab, and rituximab (CFAR) over a 5-day period each month for 6 months. This treatment regimen drastically reduced the T-cell population and virtually eliminated circulating B cells in patients with CLL. To monitor for leukemia recurrence, blood samples were evaluated for VLR39-reactive CD5<sup>+</sup>/CD19<sup>+</sup> B cells by flow cytometric analysis (Fig. 3). B cells were undetectable 4 months after the beginning of therapy, and thereafter progressive reconstitution of both T and B lymphocytes was observed (Fig. 4). CD5<sup>+</sup>/CD19<sup>+</sup> cells appeared at very low frequency at 29 months after CFAR therapy and gradually increased by 56 months. The interpretation of this



**Figure 3.** Monitoring for CLL recurrence. Blood samples were periodically obtained from CLL donor after CFAR treatment and analyzed by flow cytometry for recurrence of VLR39<sup>+</sup>/CD5<sup>+</sup>/CD19<sup>+</sup> cells. Each time point is calculated as months after the end of the CFAR treatment regimen. VLR39 binding (black line) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). Shown are anti-CD5 and anti-CD19 staining of CLL donor MNCs after treatment (left column) and histograms of VLR39 binding to CLL donor lymphocytes gated on CD5<sup>+</sup> (middle column) or CD5<sup>-</sup>/CD19<sup>+</sup> B cells (right column).

development was unclear, given that some B cells in healthy individuals express CD5. However, VLR39<sup>+</sup> B cells were detectable 51 months after therapy in the CD5<sup>+</sup> B-cell population (Fig. 3), when neither the lymphocyte count nor proportion of CD5<sup>+</sup> B cells were significantly increased (Fig. 4). To test whether the VLR39<sup>+</sup> cells represented recurrence of the leukemic clone, we purified both VLR39<sup>+</sup>/CD5<sup>+</sup> and VLR39<sup>-</sup>/CD5<sup>-</sup> B-cell populations from the CLL donor for sequence analysis of the *VH* region (Supplementary Fig. S4). The *IGHV* gene sequence of the VLR39<sup>+</sup>/CD5<sup>+</sup> sorted B cells proved to be identical to that of the original leukemic clone, whereas the VLR39<sup>-</sup>/CD5<sup>-</sup> sorted B cells did not yield a single uniform sequence, indicating their polyclonal nature. The VLR39 idiotope thus provided an early indication of the reemergence of the CLL clone in this patient. When the same blood samples were assayed using quantitative real-time PCR (qPCR) for the CLL donor HCDR3 transcripts, the emergence of the leukemia clone was first detectable at 51 months, in agreement with the VLR39 staining results (Supplementary Table S2).



**Figure 4.** B-cell and absolute lymphocyte count of patient after CFAR therapy treatment. The blood counts from the CLL donor's clinical laboratory studies were used to plot absolute lymphocyte counts over time (black circle and line, left y-axis). We calculated the proportions of CD5<sup>-</sup> B cells (gray bars) and CD5<sup>+</sup> B cells (black bars) from our own flow cytometric studies and plotted them as a percentage of total lymphocytes over time (right y-axis).

There has been longstanding interest in using the tumor-specific nature of anti-Id antibodies for monitoring and treating B-cell malignancies (25). One barrier to implementation of this strategy is the arduous nature of generating anti-Id antibodies for each individual patient via hybridoma generation and screening, which can take up to several months after immunizing mice with patient cells. The single-chain polypeptide nature of the VLRB protein offers a technologic advantage in that the VLRB sequence can be cloned, expressed, and screened within a matter of days after lamprey immunization with patient cells (4). Selection of VLRB antibodies using high-throughput antibody display technologies allows for even more

efficient screening of Id-specificity (15). The precise specificities of VLRB antibodies may also facilitate the identification, isolation, and characterization of the leukemic clones in patients with B-cell malignancies, particularly those with minimal residual disease. Lamprey LRR-based anti-Id antibodies thus offer a complementary biologic tool to the classical Ig-based anti-Id antibodies for monitoring the therapy for patients with CLL and other B-lineage malignancies.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** H. Nakahara, M.D. Cooper

**Development of methodology:** H. Nakahara, B.R. Herrin, R. Catera, M.D. Cooper

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H. Nakahara, M.N. Alder, X.-J. Yan, N. Chiorazzi, M.D. Cooper

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H. Nakahara, B.R. Herrin, M.D. Cooper

**Writing, review, and/or revision of the manuscript:** H. Nakahara, B.R. Herrin, N. Chiorazzi, M.D. Cooper

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** R. Catera, N. Chiorazzi, X.-J. Yan, M.D. Cooper

**Study supervision:** M.D. Cooper

#### Acknowledgments

The authors thank Dr. Larry G. Gartland at the University of Alabama at Birmingham and Sommer Durham at the Emory University School of Medicine Flow Cytometry Core Facility for assistance with FACS studies.

#### Grant Support

This work was supported in part by NIAID/NIH grants R01 AI072435 and R37 AI039816 (to M.D. Cooper) and NCI/NIH grant RO1 CA081554 (to N. Chiorazzi).

Received May 3, 2013; revised July 29, 2013; accepted July 30, 2013; published OnlineFirst August 5, 2013.

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*Cancer Immunol Res* 2013;1:223-228. Published OnlineFirst August 5, 2013.

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