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Ancient Lamprey VLR Antibodies as Tumor Diagnostic and Tumor Targeting Reagents

By

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Graduate Division of Biological and Biomedical Sciences
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An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Immunology and Molecular Pathogenesis

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ABSTRACT

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Jawless vertebrates (lamprey and hagfish) possess an unusual adaptive immune system that lacks conventional Ig/TCR genes used by all other vertebrate species for antigen recognition. Instead, jawless vertebrates use leucine-rich repeats (LRR) to generate three types of variable lymphocyte receptors (VLR): VLRA and VLRB found on T-like cells, and VLRB found on B-like cells. In response to immunization, VLRB cells proliferate and differentiate into VLRB antibody-secreting plasmacytes. The potential VLRB antibody repertoire is estimated to be greater than 1014 unique VLR clones, which are generated through a gene conversion-like process that replaces the non-coding segments within the incomplete germline VLR gene with randomly selected sequence diverse LRR subunits. Given the 500 million years of evolution separating jawless vertebrates from all other vertebrates, VLR should be able to access novel epitopes that are forbidden to conventional Ig due to self-tolerance.

In search of novel tumor-specific epitopes, we immunized lampreys with B cell leukemia clones from patients with B cell chronic lymphocytic leukemia (CLL) or mouse BCL1 leukemia to generate recombinant monoclonal VLRB antibody libraries, which were then screened for tumor-specificity. From the CLL-immunized library, we identified an antibody, VLR39, which was specific for the donor CLL cells and recognized the heavy chain variable region (VH) complementarity determining region 3 (CDR3) of the B-cell receptor (BCR). Using this antibody to monitor the CLL donor after chemoimmunotherapy-induced remission, we detected the recurrence of the leukemic clone before significant increase in lymphocyte count or CD5+ B cells. From the BCL1-immunized library, we identified an antibody, VLR-C8, which was specific for the BCL1 clones and also found to recognize the VH/VL CDR3 of the BCR.

Lamprey antibodies exhibit exquisite specificity for a protein epitopes, which in this case was the signature VH/VL CDR3 sequence of B cell leukemia clones, and offer a rapid strategy for generating anti-idiotype antibodies for early detection of leukemia recurrence and may potentially be used as a tumor targeting reagent.

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Lastly, I dedicate this work to my late father. You bestowed on me a name meaning "expansive knowledge", which seems particularly fitting given my scholarly career aspirations. I'll be doing my best to live up to it and make you proud. Miss you.

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LIST OF ABBREVIATIONS

7-AAD:	7-aminoactinomycin D
ACK:	ammonium chloride/potassium
ADA:	anti-drug antibodies
ADCC:	antibody-dependent cellular cytotoxicity
AID:	activation-induced cytidine deaminase
APC:	allophycocyanin
APOBEC:	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
BclA:	Bacillus collagen-like protein of anthracis
BCR:	B cell receptor
BG-H:	blood group H disaccharide
BG-H3:	blood group H type 3 trisaccharide
BiTe:	Bispecific T cell Engager
CD:	cluster of differentiation
CDA:	cytidine deaminase
CDC:	complement-dependent cytotoxicity
cDNA:	complementary DNA
CDR:	complementarity determining region
CFAR:	cyclophosphamide, fludarabine, alemtuzumab, and rituximab
CH:	heavy chain constant domain
CLL:	B cell chronic lymphocytic leukemia
CTD:	C-terminal domain
DARPin:	designed ankyrin repeat proteins
DMEM:	Dulbecco’s Modified Eagle’s Medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
EBV:	Epstein-Barr virus
EIA:	enzyme immunoassay
ELISA:	enzyme-linked immunosorbent assay
EpCAM:	epithelial cell adhesion molecule
Fab:	fragment antigen-binding
FBS:	fetal bovine serum
Fc:	fragment crystallizable
FDA:	U.S. Food and Drug Administration
FITC:	fluorescein isothiocyanate
FR:	framework region
GalNAc:	N-acetylgalactosamine
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GlcNAc:	N-acetylglucosamine
GPI:	glycosyl-phosphatidyl inositol
HCabs:	heavy-chain antibodies
HCDR:	Ig heavy chain CDR
HEK:	human embryonic kidney
HEL:	hen egg lysozyme
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Id:	idiotype
Ig:	immunoglobulin
IRES:	internal ribosome entry site
LACI:	lipoprotein-associated coagulation inhibitor
LacNAc:	N-acetyllactosamine
LCDR:	Ig light chain CDR
LDL:	low-density lipoprotein
LRR:	leucine-rich repeat
LRRNT:	N-terminal LRR
LRRCT:	C-terminal LRR
mAb:	monoclonal antibody
MEM:	minimum essential medium
MFI:	mean fluorescence intensity
MHC:	major histocompatibility complex
MNCs:	mononuclear blood cells
Neu5Ac:	N-acetylneuraminic acid
Ni-NTA:	nickel-nitrilotriacetic acid
NK:	natural killer
PBMC:	peripheral blood mononuclear cell
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PE:	phycoerythrin
PECy7:	phycoerythrin-cyanine7 dye
PEI:	polyethylenimine
Poly(I:C):	polyinosinic:polycytidylic acid
qPCR:	quantitative real-time PCR
RAG:	recombination-activating gene
RIA:	radio immunoassay
RNA:	ribonucleic acid
RPMI:	Roswell Park Memorial Institute
RT-PCR:	reverse transcription PCR
scFv:	single-chain variable fragment
TBS:	Tris buffered saline and 0.05% Tween-20
TCR:	T cell receptor
TFα:	Thomson-Friedenreich antigen
TFPI:	tissue factor pathway inhibitor
Tn:	Thomsen-nouvelle antigen
TNF:	tumor necrosis factor
V(D)J:	variable, diversity, and joining
VH:	heavy chain variable domain
VHH:	variable domain of heavy-chain antibodies
VL:	light chain variable domain
VLR:	variable lymphocyte receptor
V-NAR:	variable domain of new antigen receptor
vWF:	von Willebrand factor
WT:	wild type

CHAPTER 1

INTRODUCTION

Part I: Finding Inspiration in the Immune System

Vaccination

The understanding of the immune system and the ability to manipulate it have proven invaluable in humanity's fight against disease and illness. Even as far back as 11th century China, it was recognized that survivors of smallpox infection became resistant to re-infection. Thus, it became common practice to intentionally inoculate individuals with material from active smallpox lesions in a process known as variolation, which resulted in a milder smallpox infection that conferred protection against naturally acquired smallpox infection. However, the presence of the smallpox virus in the inoculum itself carried the risk of causing severe and lethal infection in a minority of variolated individuals (1). Seven centuries later on the other side of the world, Edward Jenner observed that individuals with a past history of cowpox infection resisted the effects of variolation or natural smallpox infection. This led Jenner to experimentally inject children with cowpox pustules, which conferred protection from smallpox challenge (2). Not only did Jenner invent a safer immunization technique by using non-lethal cowpox, but his work directly led to the wide acceptance of vaccination as a method to prevent infectious disease (3). In a testament to the effectiveness of vaccination, the World Health

Organization announced the eradication of smallpox in 1980 after a successful global vaccination effort (4).

Serum Thereapy

Continual tinkering with the immune system would yield another important fruit at the end of the 19th century, when Emil von Behring and Shibasaburo Kitasato demonstrated that the immune system could be used not only to prevent, but to treat disease by transfer of passive immunity. In their 1890 paper, “On the realization of immunity in diphtheria and tetanus in animals” originally published in German (5–7), they observed that animals immunized with tetanus and diphtheria carried toxin-neutralizing properties in the cell-free fraction of the blood (serum), and that the immune serum could be preemptively transferred into un-immunized animals to protect against infection, as well as to treat infected animals. Von Behring adapted and perfected the technique for human use, and was awarded the first ever Nobel Prize in Physiology or Medicine in 1901 "for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths" (8).

Working with von Behring on the standardized production of anti-diphtheria serum, Paul Ehrlich developed effective immunization protocols in cattle to achieve high antibody titers (9,10). Shortly thereafter in 1897, Ehrlich proposed a remarkably prescient theory to explain the mechanism for anti-toxin production. In his side-chain/receptor theory, cells expressed a set of receptors with different structures and binding

capabilities, which can interact with toxins in a precise and specific manner similar to the lock and key model of enzyme/substrate interaction. In response to binding the toxin, the cell increases the production of that specific receptor and sheds the excess into the blood stream as anti-toxin (10–13). Though experimental proof would not come for another half century, Ehrlich essentially predicted the existence of B cells expressing surface immunoglobulin receptors that could differentiate into antibody secreting plasma cells in response to antigen stimulation. For this incredible feat of prognostication, Ehrlich deservedly shared the 1908 Nobel Prize in Physiology or Medicine with Élie Metchnikoff for laying the theoretical foundation of immunology (14). The specificity of the antigen-antibody interaction inspired Ehrlich to coin the term “magic bullet” to describe an optimal therapeutic agent that could target pathogens and tumors while leaving the host cells unharmed (12,13).

Monoclonal Antibodies

Serum therapy came close, but was not precise enough to be a true magic bullet. Patients could develop serum sickness several days after injection due to the non-human origin of the anti-diphtheria serum (6). Switching to human serum donors mitigated this problem somewhat, but a heterologous mixture of antibodies that may target auto-antigens or circulating microbial antigens could still cause adverse effects (15). The observation that myeloma cells produce homologous, monoclonal antibodies with restricted specificity offered promise, but it was impractical to experimentally induce myelomas and screen them hoping to find the desired specificity by chance (16). Finally in 1975, Kohler and Milstein published a method for generating monoclonal antibodies

with specificities directed against the target immunogen of choice (17). By fusing splenic cells from an immunized mouse to a myeloma cell line, they could preferentially immortalize the antigen-specific B cells that responded to the immunogen, which could then be screened for the desired specificity and recovered. Kohler and Milstein were jointly recognized with the 1984 Nobel Prize in Physiology or Medicine for "…the discovery of the principle for production of monoclonal antibodies" (18).

It only took 11 years from the publication of the hybridoma method before the U.S. Food and Drug Administration approved the first monoclonal antibody for clinical use. This mouse anti-CD3 monoclonal antibody, muromonab, was approved for use as an immunosuppressive agent to prevent transplant rejection (19,20). Again, as seen before in serum therapy, muromonab and other monoclonals being investigated for clinical use at the time had side effects associated with immunogenicity owing to their non-human origin. In addition, the Fc portion of the mouse antibody did not interact efficiently with the human Fc receptor, limiting its effector functions and shortening its serum half-life (21). The first attempt at circumventing these issues was to construct chimeric antibodies that joined the antigen-binding mouse Ig variable region with the human Ig constant region containing the Fc region, which would allow interaction with the human Fc receptor (22). Immunogenicity was further reduced with humanized antibodies that replaced the antigen-binding CDR loops in a human antibody with the antigen-binding CDR loops from a mouse antibody (23,24). Eventually, deriving fully human antibodies from transgenic mice containing the human heavy and light chain genes (25,26) and *in vitro* screening of human antibody display libraries (27,28) became a possibility. By 2012, the total U.S. sales of monoclonal antibody drugs recorded a staggering 24.6 billion

dollars (29). As of this writing in 2015, there are 46 different monoclonals in regulatory review or approved for sales in the US or EU, many of them full-length human IgG antibodies (30).

Von Behring and Kitasato had ended their 1890 paper with a quote from Goethe: 'Blut ist ein ganz besonderer Saft' translated from the original German as "blood is a quite peculiar juice" (7). Less than a century after the discovery of serum therapy, the efforts of immunologists to unlock the exact nature of this 'peculiar juice' had brought about a game changing technology to produce magic bullets, in principle, at will.

Part II: Improving on Nature

Limitations of the IgG Format

The qualities that make a full-length human IgG antibody a great magic bullet is its antigen-specificity, bivalent structure which provides high avidity binding, reduced immunogenicity due to its human origin, and Fc receptor interactions which confers immune effector functions and increased serum half-life (31,32). However, the physiochemical properties inherent to the IgG antibody can be a disadvantage for certain applications. In the case of cancer therapy, the 150 kDa IgG antibody is too large for efficient tissue penetration, limiting its effectiveness at targeting solid tumors (33). Fc receptor-bearing cells may be non-specifically activated by the Fc region of the antibody, which can potentially trigger a cytokine storm and other undesirable effects (32). For applications where the antibody is conjugated to imaging/therapeutic agents, the Fc-mediated effector functions are unnecessary and become a hindrance; the increased serum half-life of the antibody through neonatal Fc receptor recycling and non-specific

uptake by Fc receptor-bearing cells reduces target specificity and delays bloodstream clearance, which in turn leads to poor imaging contrast and exposure of healthy tissue to the toxic drug/radionuclide (33). From a manufacturing standpoint, the complexity of the IgG molecule with its four polypeptides (two pairs of heavy and light chain), the disulfide bonds crucial for stability, and glycosylation necessary for Fc function are all factors that require an eukaryotic expression system, which is costly and inefficient compared to a prokaryotic expression system (31,34).

Engineered Ig Domains

These limitations have been addressed to a certain extent by the manipulation and engineering of the Ig domain. Antibody fragments can be generated by proteolysis of full-length antibodies, or cloning and expression of shortened Ig genes that encode a single polypeptide as Fab, scFv, and even isolated single Ig domains (32,34). The smallest of this category are the single Ig domains that are $1/10^{th}$ the size of full-length antibodies, making them attractive for use in imaging and tumor targeting applications. The shortened, single-chain structure of antibody fragments make them more amenable to library display than the heterodimeric Ig chains, and allows improved expression efficiency in bacteria, albeit in the oxidative environment of the periplasm due to the importance of the disulfide bond for protein stability (32,35,36). Two scFv with different specificities can be linked together to produce bispecific molecules (32) which have had promising clinical results. The FDA recently approved blinatumomab (MT103), a Bispecific T cell Engager (BiTe) antibody with specificity to both CD3/CD19, for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor

acute lymphoblastic leukemia (37). Blinatumomab can use the scFv-CD3 arm to engage cytotoxic T cells regardless of TCR specificity, and direct them towards killing malignant B cells bound by the scFv-CD19 arm (37,38). Another BiTe antibody with EpCAM/CD3-specificity (MT110) (39) and a bispecific tandem diabody (TandAb) with CD30/CD16A-specificity (AFM13) (40) have completed Phase I clinical trials.

The discovery of animals with natural single domain antibodies offers an intriguing possibility of generating smaller antibodies that have undergone "quality control" through millions of years of natural evolution. The *Camelidae* (camels, llamas, and alpacas) (41) and cartilaginous fish (nurse sharks, wobbegong, and ratfish) (42–44) all possess a subset of antibodies consisting of a pair of single heavy chains missing the CH1 domain and lacking the light chain, known as heavy-chain antibodies (HCAbs) (45). The antigen-binding domain of both the camelid and shark HCAbs, known as the VHH and V-NAR respectively, demonstrate superior solubility, stability and lack of self-aggregation compared to human single domain antibodies (32). A unique feature of these single domain antibodies are the long CDR loops that allow access to cleft epitopes (46), such as the catalytic site of enzymes and ligand-binding site of receptors (45). In contrast, the CDR loops of conventional VH/VL mouse antibodies are generally unable to bind to concave epitopes (47). Several nanobodies based on the *Camelidae* VHH domain are currently undergoing Phase I/II clinical trials with excellent safety and immunogenicity records thus far (48). A divalent nanobody comprised of two anti-von Willebrand factor (vWF) VHH joined by a 3-alanine linker, caplacizumab (ALX-0081), is being evaluated for treatment of acquired thrombotic thrombocytopenic purpura (49). Ozoralizumab (ATN-103) for rheumatoid arthritis is a trivalent nanobody consisting of

two anti-TNF-α VHH and one anti-albumin VHH, which can utilize the targeting properties of albumin for improve pharmacokinetics (50).

Even with these stepwise innovations, antibodies and their derivatives are inherently reliant on their disulfide bridges for structural stability and much optimization remains for efficient prokaryotic expression (51–53). In addition, the complex slew of patents governing the generation, screening, and production of antibodies and their derivatives make them very expensive for both patients and manufacturers (54–57).

Non-Ig Protein Scaffolds

Instead of iterating on the Ig domain, great effort has been made to use alternative protein scaffolds as a starting point for developing antigen-specific, high affinity-binding molecules. Focusing on *in vivo* applications, ideal features include: small size to enhance tissue penetration, thermochemical stability, lack of disulfide bonds for cost-effective production by prokaryotic expression or chemical synthesis; versatility that allows pharmacokinetic alteration (short half-life for imaging versus increased half-life for therapy), payload conjugation, multi-specificity and fusion constructs (36,58). Most importantly, protein scaffolds need to feature a binding surface amenable to the introduction of random sequence diversity to generate a library, which can then be screened for binding to the target of interest (36,38).

In 2009, Ecallantide (Kalbitor/ DX88), a potent and specific kallikrein inhibitor used for the treatment of hereditary angioedema, became the first engineered non-Ig protein scaffold to meet FDA approval for therapy (20,38,59). The lead compound, DX88, was discovered by phage display of the 7 kDA Kunitz domain from the human

lipoprotein-associated coagulation inhibitor (LACI), also known as tissue factor pathway inhibitor (TFPI), and screening for kallikrein binding and inhibitor activity (60,61). Many more non-Ig protein scaffolds are under investigation and development (31,34,36,48,57,58): Adnectins derived from a 94 amino acid type III fibronectin domain, Avimers from a 35 amino acid repeating motif of the LDL receptor Domain A, Affibodies from the 58 amino acid synthetic Z domain of *Staphylococcus aureus* Protein A, DARPins from ankyrin repeat proteins comprised of 67 amino acids plus a 33 amino acid repeating motif, and Anticalins from the 160–180 amino acid lipocalins (20,38,48).

The relative advantages offered by non-Ig protein scaffolds is offset by the risk for immunogenicity due to their synthetic origins (62–64), though none have been reported to cause severe adverse reactions or anti-drug antibody in Phase I clinical trials to date (20,65–69). The non-Ig protein scaffolds are even smaller than the 15 kDa antibody fragments, which may explain their lack of immunogenicity due to rapid clearance. Nevertheless, even with comparative advantage in some applications over traditional antibodies, each protein scaffold will have their own limitations dictated by the demands of the anticipated downstream application.

Part III: The Agnathan Adaptive Immune System

Non-Ig Adaptive Immune System

The discovery and characterization of the non-Ig antibodies used by jawless vertebrates (agnathans) offers fresh inspiration for producing novel antigen-specific binding molecules. Lamprey and hagfish represent the only extant lineage of agnathans,

which split off from the jawed vertebrates (gnathostomes) nearly 500 million years ago in the Cambrian period (70–75). Historically, there was strong suggestive evidence for the existence of an adaptive immune system in the lamprey: the production of specific agglutinins and lymphoid proliferation in response to immunization, accelerated rejection of secondary allografts, and delayed-type hypersensitivity reactions (76–81). Similar, though limited evidence were also observed in hagfish (82–87). However, the agglutinins of lamprey and hagfish did not resemble any known gnathostome Ig by biochemical analysis (78,85,88), and transcriptome analysis of lamprey and hagfish leukocytes failed to identify key elements of adaptive immunity found in all gnathostomes (Ig, TCR, RAG1/2, and MHC class I/II) (89–91).

Variable Lymphocyte Receptors

The mystery was finally resolved in 2004 with the discovery of a diverse set of leucine-rich repeat (LRR) transcripts expressed in the activated lymphocyte population, which were dubbed variable lymphocyte receptors (VLR) (92). The VLR transcripts were exclusively expressed in the lymphocyte population, and comprised of an invariant signal peptide, followed by an N-terminal LRR (LRRNT), a variable number of sequence diverse LRR modules, a connecting peptide, C-terminal LRR (LRRCT), and an invariant threonine/proline-rich stalk containing a glycosyl-phosphatidyl-inositol (GPI)-anchor site and hydrophobic tail. The germline VLR gene is incomplete; it contains two non-coding intervening sequences sandwiched between three partial segments of the 5’ LRRNT, 5’ LRRCT, and 3’ LRRCT. To assemble a mature VLR gene, numerous LRR cassettes both upstream and downstream of the incomplete germline VLR gene serve as template

donors; they can be randomly and sequentially copied in-frame to replace the non-coding intervening sequences in a gene conversion-like process thought to be mediated by two lamprey orthologues of the AID-APOBEC family DNA cytosine-deaminase, CDA1 and CDA2 (93). VLR gene assembly occurs in monoallelic fashion, generating a clonally diverse population of lymphocytes that each express a single, unique VLR gene (92,94,95). Based on computational analysis of individual LRR modules found within mature VLR gene sequences, the potential VLR repertoire is calculated to be greater than 10^{14} unique clones, which is comparable to the estimated size of the mammalian antibody repertoire (94).

Three different VLR types have been identified in the lamprey (96–98) and hagfish (99). Each individual lymphocyte only expresses one of the three VLR types. The first VLR discovered was the VLRB, which is expressed as a cell surface receptor on B-like cells generated in the hematopoietic organs (typholosole and kidneys), allowing them to respond to antigen stimulation by proliferating and differentiating into plasma cells that secrete multimeric VLRB antibodies (100,101). On the other hand, VLRA and VLRC are solely expressed as cell surface receptors on T-like cells generated in thymus-like structures in the gills designated as the thymoid (98,102). VLRA cells and VLRC cells exhibit a dichotomous gene expression pattern that is remarkably similar to that of α/β and γ/δ T cells, both of which respond to stimulation by phytohemagglutinin or poly(I:C) stimulation, respectively (98,103–106).

VLRB protein and structure

Before the enormous potential of the VLRB antibody repertoire could be utilized for the generation of reagents with novel binding specificities, the first technical issue that needed to be overcome was in how to produce monoclonal VLRB antibodies. The lack of a cell culture system nor a method for immortalizing lamprey lymphocytes meant that the classic immunological methods for isolating and generating a monoclonal antibody producing clone would not be feasible. Fortunately, advances in recombinant DNA technology since the days of Kohler and Milstein, and the single polypeptide nature of the VLRB gene meant that it was possible to directly clone the somatically rearranged VLRB cDNA from individual lymphocytes and express them in HEK-293T cells (101) or yeast surface display libraries (107). This allowed for VLRB clones to be screened for the desired binding-specificity. Using both immunized and non-immunized lamprey lymphocytes as the cDNA source, monoclonal VLRB antibodies to numerous types of protein and carbohydrate antigens have been identified. These include the BclA protein of *Bacillus anthracis* exposporium (101,108), the H-trisaccharide of human blood group O erythrocytes (109), hen egg lysozyme (110), human CD5 (111), the BCR idiotope of human B cell chronic lymphocytic leukemia cells (112), the Thomsen-Friedenreich (TFα) pancarcinoma carbohydrate antigen (113) and others (107,114).

Structural data on the VLRB protein have been obtained by X-ray crystallography analysis of monoclonal VLRB antibodies in complex with their cognate antigens: anti-H-trisaccharide (RBC36) (109), anti-HEL (VLRB.2D) (110), anti-BclA (VLR4) (108), and anti-TFα disaccharide (aGPA.23) (113). The VLRB protein shares the characteristic crescent-shaped, solenoidal form with other LRR family proteins (109,110,113,115). The “LxxLxLx” motifs in the variable LRR subunits form parallel B-strands on the concave

surface, where antigen contact occurs. There is an extended loop from the C-terminal LRR that extends back towards the concave surface and provides another crucial point of antigen contact (108–110,113). The VLR is stabilized by a hydrophobic core comprised of conserved leucines and phenylalanines, capped off by the LRRNT and LRRCT regions at both ends. Additional stability comes from two disulfide bridges formed by four conserved cysteines each at the LRRNT and LRRCT (109,110,113,115). The result is a remarkably stable protein that can withstand harsh pH and temperature extremes, requiring incubation at 70°C for 1 h before antigen binding activity is lost (101).

The VLRB antibodies are secreted as multimers composed of identical subunits organized as four to five sets of dimmers that are connected by disulfide-linkage at the ends of their stalk region (100,101). Individual VLRB monomers often possess relatively low affinity, in the micromolar range, but the full size VLRB multimers display high avidity binding due to their multivalency (101,110). As an example, the anti-BclA VLRB mAb (VLR4) can agglutinate *B. anthracis* spores at a 1000-fold higher dilution than can a high-affinity anti-BclA mouse IgG2b mAb (EA2-1) (101,116). It is possible to generate high affinity VLRB clones in the nanomolar range by using error-prone PCR to introduce mutations in the VLRB diversity region (107,110).

Antigen-specificity of VLRB antibodies

VLRB antibodies exhibit exquisite specificity in that they are capable of discriminating between very similar epitopes. Such is the case with anti-BclA VLR4, which distinguishes between the very similar BclA sequences of *Bacillus anthracis*, *Bacillus cereus* T, and *Bacillus thuringiensis* (subsp. Kurstaki) (101,108). By

comparison, Ig-based monoclonal antibodies generated against the BclA of *Bacillus anthracis* are typically cross-reactive with related *Bacillus* strains (117,118). In this case, the VLR4 antibody recognizes a *Bacillus anthracis*-specific glycosylation pattern on the BclA protein (108).

Conventional antibodies (119) and most lectins (120) directed against the TFα disaccharide were of inferior affinity and specificity in comparison to the anti-TFα VLRB antibody, aGPA.23 (114). Much like aGPA.23, the most promising anti-TFα mouse antibody, JAA-F11, can distinguish TFα [Galβ1-3GalNAcα] from TFβ [Galβ1-3GalNAcβ] (121). However, JAA-F11, reacts with a wider spectrum of glycan structures, such as the core 2 trisaccharide [Galβ1-3(GlcNAcβ1-6)GalNAcα], 6-Sialyl TFα [Galβ1-3(Neu5Acβ2-6)GalNAcα], and 6-LacNAc-Tn [Galβ1-4GlcNAcβ1-6GalNAcα] whereas aGPA.23 is more precise in that it mainly recognizes fucosylated TFα (BG-H3 [Fucα1-2Galβ1-3GalNAcα] and BG-H [Fucα1-2Galβ]) and serine-linked TFα (114,122).

The exquisite specificities of VLRB antibodies may reflect the structural rigidity of the LRR β-strands comprising the concave antigen-binding surface, in contrast to the induced-fit conformational changes that allow the CDR loops of conventional antibodies to bind multiple antigens (75). However, this VLRB rigidity is tempered by the flexible, highly variable LRRCT loop which make important antigen contacts (108–110,113). Notably, the LRRCT loop of the anti-HEL VLRB.2D is inserted into the catalytic cleft of HEL (110) much like the extended CDR loops of the single chain camelid VHH (46) and shark IgNAR (123) anti-HEL antibodies. Structural studies of mouse anti-HEL antibodies in complex with HEL show that they typically recognize planar epitopes away from the catalytic cleft (47).

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Another factor that may contribute to the unusual specificity of VLRB is the evolutionary distance between agnathans and gnathostomes. This predicts that VLRB antibodies should be inducible that can recognize mammalian antigens forbidden to conventional Ig-based antibodies by immune tolerance. During B cell development in mammals, self tolerance mechanisms eliminate or inactivate self-reactive B cell clones to forestall development of autoimmunity (3). The flipside of this safety measure is the exclusion of some V(D)J gene permutations from the antibody repertoire, and the consequent inability to use those antibodies to recognize epitopes that mimic host antigens. The VLRB antibodies should thus be able to access antigenic blindspots in the Ig antibody repertoire.

The biotechnology niche occupied by VLRB

VLRB antibodies are uniquely positioned between engineered Ig domains and non-Ig protein scaffolds as a technology for producing specific-binding molecules. Compared to engineered Ig domains, the VLRB antibodies can potentially recognize novel antigens due to lack of tolerance constraints for mammalian antigens, and the unique structural basis for their antigen-binding via their rigid, but variable LRR β-strand surface, and the flexible, hypervariable LRRCT loop (108–110,113). The specificity of VLRB antibodies for glycan determinants (108,109,113) make them an attractive option for generating new glycan-binding reagents, especially in light of the estimated 7000 glycan determinants in the human glycome and the current paucity of glycan-specific reagents to study them (124).

While non-Ig protein scaffolds and VLRB antibodies share the potential for recognizing novel epitopes due to their structural difference from conventional antibodies, non-Ig protein scaffolds by definition are derived from artificial, non-immune libraries, whereas VLRB antibody libraries can be sourced from an immunized animal. A non-immune library benefits from a lack of bias in their binder repertoire and allow the discovery of novel binding-specificities, but their sheer size can pose a probabilistic challenge in isolating the desired binder (27,125). In comparison, the VLRB-containing plasma from an immunized animal can be assayed (100,126) to check for binding-specificity before screening, which greatly improves the odds of finding the VLRB clone of desired specificity and affinity.

A concern shared by both non-Ig protein scaffolds and VLRB antibodies is their immunogenicity. Despite their safety record thus far (20,65–69), it is difficult to see a path for humanizing the non-Ig protein scaffolds of synthetic origin. Fortunately, the

human Slit protein has good sequence homology with VLRB (109). This opens up the possibility of grafting the antigen-binding variable LRR residues and the highly variable LRRCT insert from the VLRB onto a Slit protein backbone to create humanized VLRB proteins.

The structural features of the antigen-binding site, evolutionary origin, and physical properties of monoclonal VLRB antibodies, all suggest that monoclonal VLRB antibodies and their engineered derivatives will prove to be useful biomedical reagents. The work in this dissertation addresses the feasibility of producing VLRB-based tumor targeting reagents, and their potential application for tumor antigen discovery, cancer diagnostics, *in vivo* imaging, and anti-tumor therapy.

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Chapter 2

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 cell chronic lymphocytic leukemia (CLL) cells 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 $VLR39^{+}$ B cells in the patient 51 months later, before significant increase in lymphocyte count or $CD5^{+}$ 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.

Introduction

The adaptive immune system of the extant jawless vertebrates, lamprey and hagfish, offers an alternative to monoclonal antibody technology as a method to produce specific binding molecules. Instead of immunoglobulin (Ig)-based antigen receptors, lampreys and hagfish use variable lymphocyte receptors (VLR) comprised of leucine-rich repeat (LRR) modules for antigen binding (1,2). Like 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 β-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 $VLRA^+$ and $VLRC^+$ lymphocytes are T-like cells that exclusively express their VLRs as cell surface molecules (9,10), whereas $VLRB^+$ 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 $>10^{15}$ 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 to 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 B cell chronic lymphocytic leukemia (CLL) patient and derivative VLRB clones were screened for CLL-specificity. Among the lamprey VLRB antibodies produced against human mononuclear blood cells (MNCs), we identified a monoclonal VLRB antibody, VLR39, that preferentially recognized the donor CLL clone. Here we describe the B cell receptor (BCR) idiotope-specificity of VLR39 and the use of this anti-idiotype antibody as a monitoring reagent for early detection of CLL recurrence.

Material and Methods

Cells and Cell Lines

Blood samples were obtained with informed consent from CLL patients 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°C in FBS supplemented with 10% DMSO. HEK-293T cells (generously provided by Dr. Tim Townes, UAB) were maintained in DMEM supplemented with 5% FBS at 37°C in 5% CO_2. B cell lines were maintained in RPMI 1640 media supplemented with 10% FBS at 37°C in 5% 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/κ monoclonal antibody (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/κ 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,

and 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 (2), received intracoelomic injections of 1×10^7 blood MNCs from a newly diagnosed CLL patient on days 0, 14 and 28, before sacrifice on day 42 for collection of buffy coat and plasma (2). All experiments were approved by 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 CLL patients 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 added before flow cytometric analysis of the cells.

Purification of monoclonal VLR39 antibody

Purified VLRB proteins were obtained by PCR cloning the VLR39 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 μg/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 10× binding buffer (500 mM NaH_2PO_4, pH 8.0, 1.5M NaCl, 100 mM 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 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.05% Tween-20) before elution with 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole, 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 previously described (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. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as previously described (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 utilizing overlap-

extension PCR with KOD Hot Start DNA polymerase (Novagen) using 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 PEI as described (4). Transfected cells were dissociated from culture plates using Cellstripper (Mediatech) and harvested 48 to 72 h post-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 profile 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°C with 5 µg/ml of recombinant Fab fragments either from the donor CLL or another CLL with unmatched V genes. Non-specific 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 CLL patients 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 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 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 CLL patients (Figure 1), lymphocytes or other types of blood cells from healthy adults, or with B cell leukemia/lymphoma lines (Figure S1). The low MFI 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 purified protein was used for subsequent analyses. Staining with purified VLR39, rather than transiently transfected HEK-293T supernatant, 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 of another CLL patient, 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 with the donor CLL Ig Fab and not with the 141 CLL Ig Fab having different *VH/VL* gene sequences (Figure 2A). Id-specificity was tested against other CLL cells selected for partial overlap of their VH sequences with the donor CLL BCR sequence (Table S1). VLR39 failed to react with tumor cells from 26 additional CLL patients (Figure S2) even though the BCRs of seven (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 three other CLL patients (1371, 336, 758, 1330) shared identical IGHJ sequences comprising the 3' end of the VH CDR3 (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 CLL patients tested were all different from each other, further 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 donor CLL BCR and two mutants (1324 HCDR3 swap and donor HCDR3 swap) were expressed by transfected cells as a single-chain variable fragment (scFv) with a myc-tag (Fig 2B) and evaluated for anti-myc and VLR39 binding by immunofluorescence flow cytometry analysis (Figure 2C). The scFv mutant, 1324 HCDR3 swap, replaces the CDR3 signature sequence of the donor CLL VH with the CDR3 signature sequence of 1324 CLL VH. The other scFv mutant, donor HCDR3 swap, replaces the CDR3 signature sequence of the 1324 CLL VH with the CDR3 signature

sequence of the donor CLL VH. The 1324 HCDR3 swap transfectants failed to stain with VLR39, whereas VLR39 and anti-myc co-staining of the donor CLL Ig and donor HCDR3 swap transfectants confirmed that the donor CLL VH CDR3 region is the VLR39 antibody recognition site.

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 five day period each month for 6 months. This treatment regimen drastically reduced the T cell population and virtually eliminated circulating B cells. To monitor for leukemia recurrence, blood samples were evaluated for VLR39-reactive $CD5^+/CD19^+$ B cells by flow cytometric analysis (Figure 3). B cells were undetectable 4 months after the beginning of therapy, and progressive reconstitution of both T and B lymphocytes was observed thereafter (Figure 4). $CD5^+/CD19^+$ cells appeared at very low frequency at 29 months after CFAR therapy, and gradually increased by 56 months. The interpretation of this development was unclear given that some B cells in healthy individuals express CD5. However, $VLR39^+$ B cells were detectable by 51 months after therapy in the $CD5^+$ B cell population (Figure 3), when neither the lymphocyte count nor proportion of $CD5^+$ B cells were significantly increased (Figure 4). To test whether the $VLR39^+$ cells represented

recurrence of the leukemic clone, we purified the VLR39^{+}/CD5^{+} and VLR39^{-}/CD5^{-} B cell populations from the CLL donor for sequence analysis of the VH region (Figure S4). The *IGHV* gene sequence of the VLR39^{+}/CD5^{+} sorted B cells proved to be identical to that of the original leukemic clone, whereas the VLR39^{-}/CD5^{-} 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). 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 technological 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 biological tool to the classical Ig-based anti-Id antibodies for monitoring the therapy of patients with CLL and other B lineage malignancies.

Figures and Legends

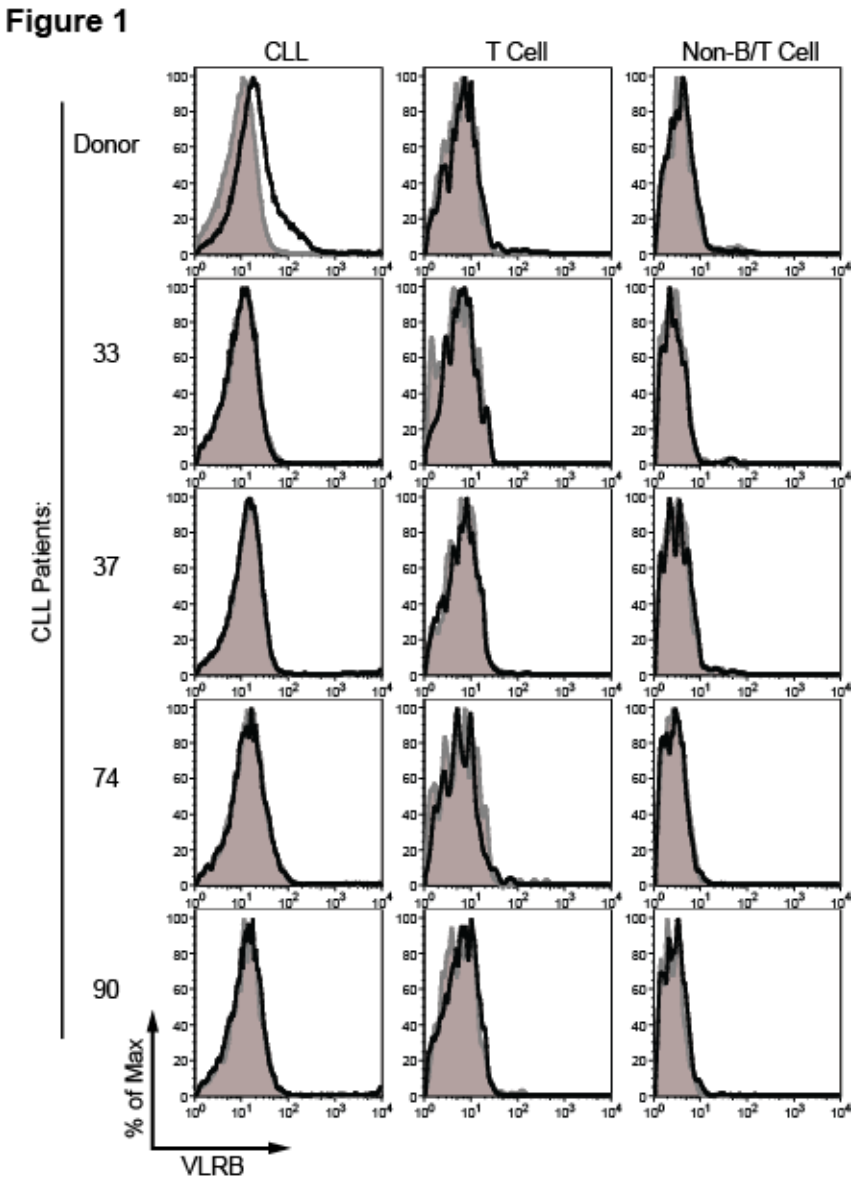

Figure 1: Flow cytometric analysis of monoclonal VLR39 reactivity. VLR39 binding (black line) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). Histogram of VLR39 binding to (A) CLL donor and four other CLL patient lymphocytes gated on CLL cells ($CD5^+/CD19^+$),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

Figure 2

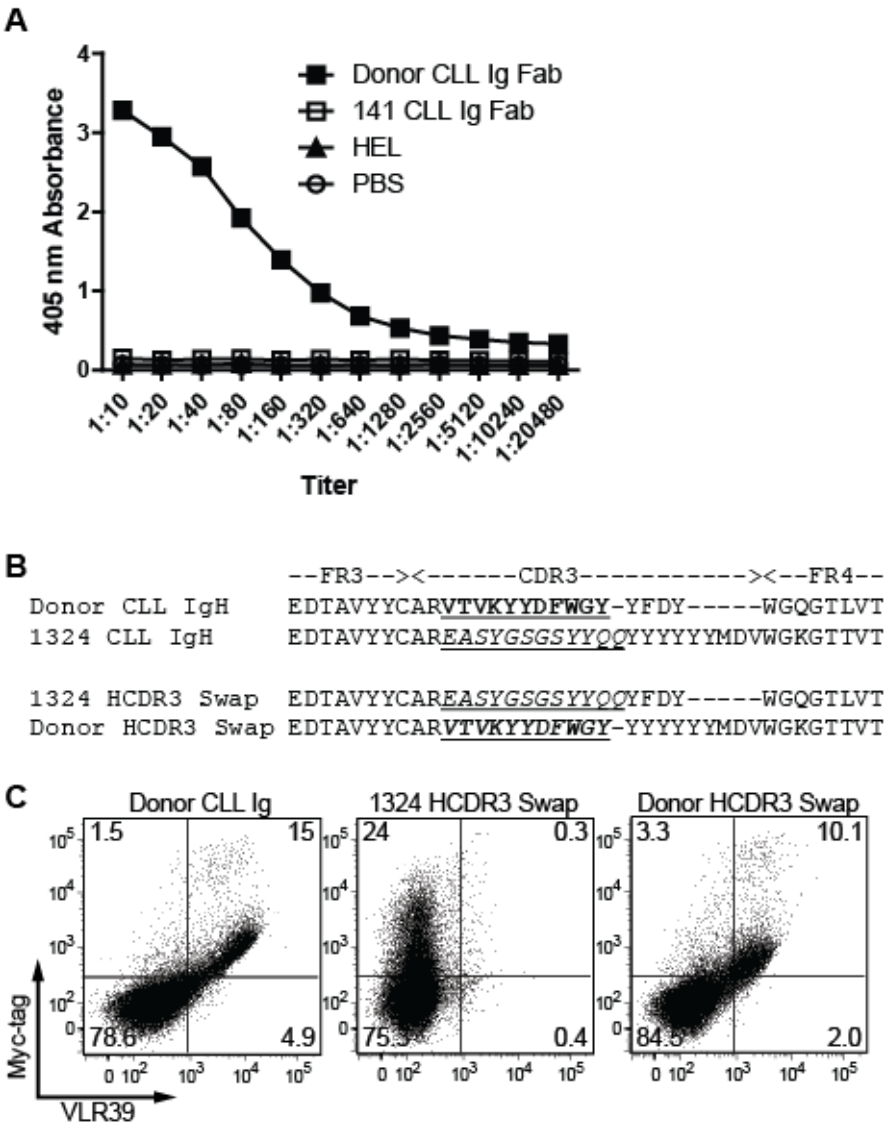

Figure 2: ELISA and flow cytometric analysis of VLR39 binding to donor CLL Igs. (A) VLR39 binding to recombinant Fab fragments containing the VH/VL genes of the CLL clone from patient donor and from another patient with unmatched VH/VL genes were compared. ELISA of VLR39 binding to donor CLL Ig Fab (black 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, underline text) and the HCDR3 signature sequences of CLL patient 1324 (italic, underline 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. Myc-tag was co-stained to measure scFv surface expression. The IgH sequences of donor CLL and 1324 CLL in panel B were provided by R. Catera and N. Chiorazzi.

Figure 3

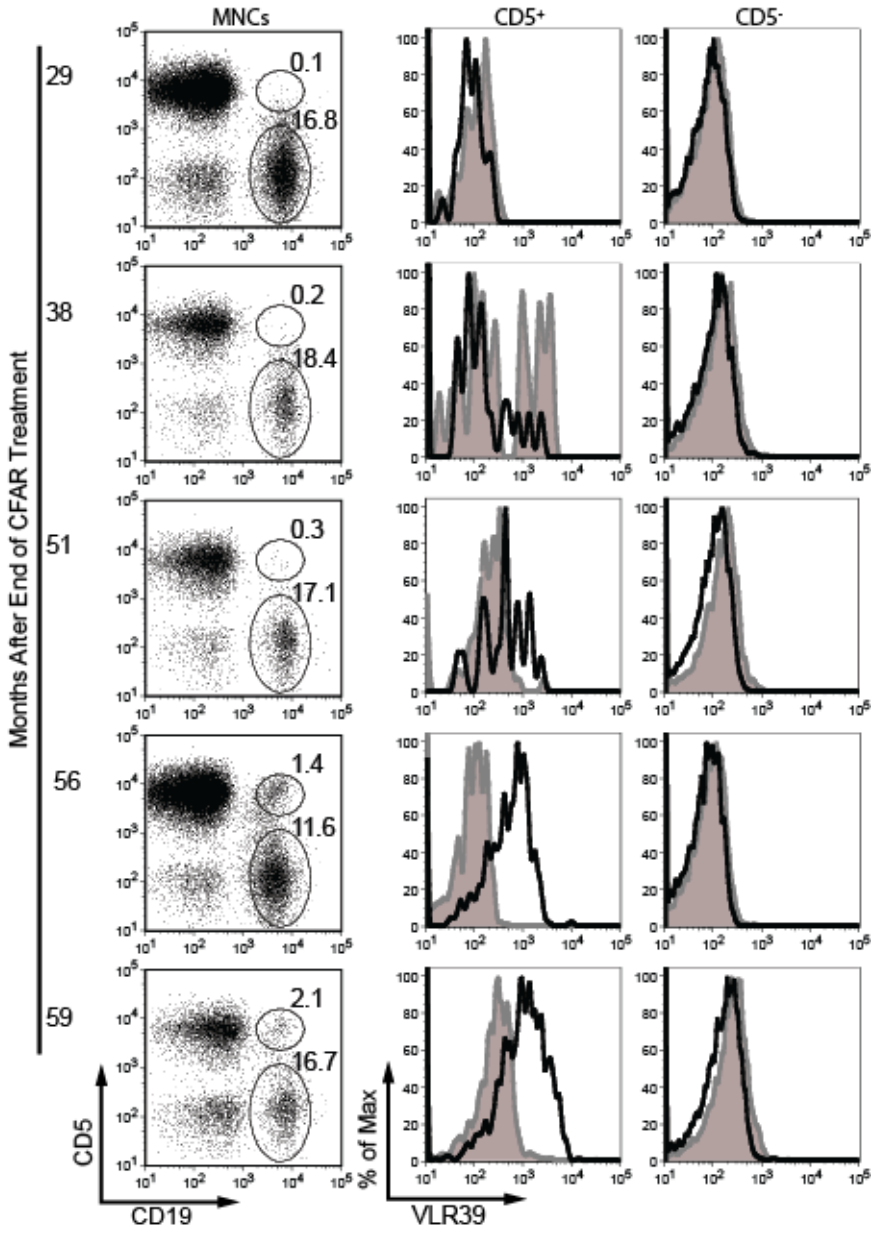

Figure 3: Monitoring for CLL recurrence. Blood samples were periodically obtained from CLL donor after treatment and analyzed by flow cytometry for recurrence of $VLR39^{+}/CD5^{+}/CD19^{+}$ cells. Each time point is calculated as months after end of the CFAR treatment regimen. VLR39 binding (black line) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). Anti-CD5 and anti-CD19 staining of CLL donor MNCs after treatment (left column). Histograms of VLR39

binding to CLL donor lymphocytes gated on $CD5^+$ (center column) or $CD5^-$ $CD19^+$ B cells (right column).

Figure 4

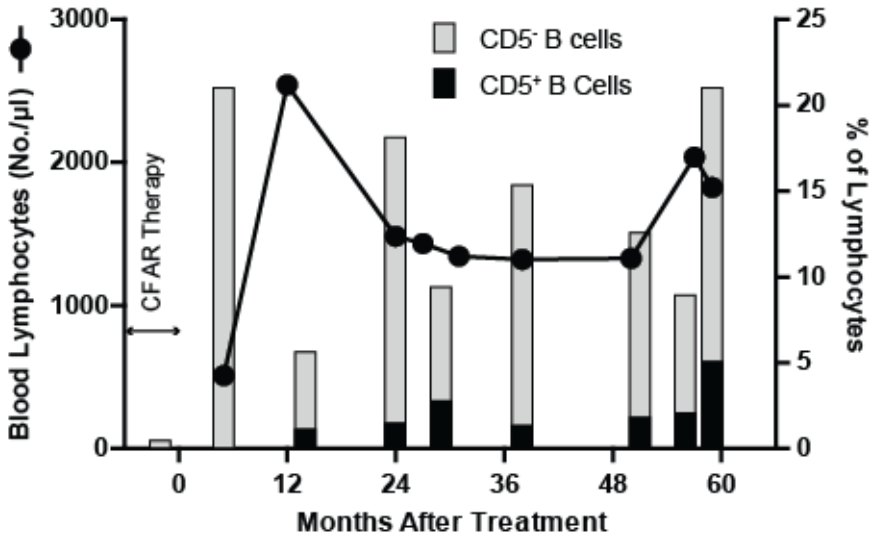

Figure 4: B cell and absolute lymphocyte count of patient after treatment. The blood counts from the CLL donor's clinical laboratory studies were used to plot absolute lymphocyte over time (black circle and line, left Y axis). We calculated the proportion of $CD5^-$ B cells (gray bars) and $CD5^+$ B cells (black bars) from our own flow cytometric studies, and plotted it as a percentage of total lymphocytes over time (right Y axis).

Supplementary Materials and Methods

Cloning of CLL donor BCR as scFv

Briefly, for the first round of PCR, the VH region was amplified using a 5' primer containing a XmaI site, CLL655_VH_FR1_XmaI_Fwd (GTCCTCGCAACTGCCCCATCCCGGGGCCCAACCAGCGATGGCCCAGGTGCAGCTGGTGCAGTCTGG) and 3' primer containing a $(Gly_4\text{-}Ser)_3$ linker sequence, CLL655_VH_scFv_Rev (CCGCCGGATCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGTGAC) with the following cycle profile: 94°C for 5 min, (94°C, 1 min; 55°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min. For VL region amplification, a 5' primer containing a $(Gly_4\text{-}Ser)_3$ linker sequence, CLL655_VL_scFv_Fwd (GGAGGCGGTTCAGGCGGAGGTGGATCCGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCC) and 3' primer containing a SalI site and FLAG tag, CLL655_VL_FR4_SalI_Rev (GAGTCATTCTCGACTGCTATGTCGACTTTATCATCATCATCTTTATAATCACGTTTGATATCCACTTTGGT) were used with the following cycle profile: 94°C for 5 min, (94°C, 1 min; 55°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min. For the second round of PCR, the scFv gene was assembled by overlap-extension based on the $(Gly_4\text{-}Ser)_3$ linker sequence homology between the VH and VL PCR products using the following cycle profile: 94°C for 5 min, (94°C, 1 min; 60°C, 1 min; and 72°C, 3 min) × 6, 72°C for 7 min. In the third round of PCR, the assembled scFv gene was PCR amplified using the outside primers, CLL655_VH_FR1_XmaI_Fwd and

CLL655_VL_FR4_SalI_Rev using the following cycle profile: 94°C for 5 min, (94°C, 1 min; 55°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min. PCR products were purified by QIAquick Gel Extraction Kit (Qiagen), digested with XmaI and SalI (New England Biolabs), and cloned into pDISPLAY vector (Invitrogen) using T4 DNA ligase (New England Biolabs). CLL donor scFv plasmid was purified from transformed NovaBlue competent cells (Novagen) with the QIAprep Spin Miniprep Kit (Qiagen).

Construction of scFv mutants

The two scFv mutants were constructed by amplifying the VH and VL regions from the wildtype CLL donor scFv plasmid utilizing overlap-extension PCR as described above, except using different primer sets for the first round of PCR. For the 1324 HCDR3 Swap scFV mutant, the VH region was amplified using a 5' primer containing a XmaI site, CLL655_VH_FR1_XmaI_Fwd (GTCCTCGCAACTGCCCCATCCCGGGGCCCAACCAGCGATGGCCCAGGTGCAGCTGGTGCAGTCTGG) and 3' primer containing the CLL1324 VH CDR3 sequence as overhang, CLL655_FR3-1324_CDR3_Rev (TTGTTGATAATAACTCCCTGAACCATAGGACGCCTCTCTCGCACAGTAATA). The VL region was amplified using a 5' primer containing the CLL1324 VH CDR3 sequence as overhang, CLL1324_CDR3-655_FR4_Fwd (GAGGCGTCCTATGGTTCAGGGAGTTATTATCAACAATACTTTGACTACTGG) and 3' primer containing a SalI site and FLAG tag, CLL655_VL_FR4_SalI_Rev (GAGTCATTCTCGACTGCTATGTCGACTTTATCATCATCATCTTTATAATCACGTTTGATATCCACTTTGGT). The 1324 HCDR3 Swap scFv gene was assembled by

overlap-extension based on the CLL1324 VH CDR3 sequence homology between the VH and VL PCR products. For the Donor HCDR3 Swap scFv mutant, the VH region was amplified using a 5' primer containing a XmaI site, CLL655_VH_FR1_XmaI_Fwd (GTCCTCGCAACTGCCCCATCCCGGGGCCCAACCAGCGATGGCCCAGGTGCAGCTGGTGCAGTCTGG) and 3' primer containing the CLL1324 VH FR4 sequence as overhang, CLL655_CDR3-1324_FR4_Rev (TGAGGAGACGGTGACCGTGGTCCCTTTGCCCCAGACGTCCATGTAGTAGTAGTAGTAGTAGTACCCCCAAAAATC). The VL region was amplified using a 5' primer containing the CLL1324 VH FR4 sequence as overhang and 5' end of a $(Gly_4\text{-}Ser)_3$ linker sequence, CLL1324_FR4-GlySer_Fwd (TACTACTACTACTACTACATGGACGTCTGGGGCAAAGGGACCACGGTCACCGTCTCCTCAGGTGGAGGCGGTTCA) and 3' primer containing a SalI site and FLAG tag, CLL655_VL_FR4_SalI_Rev (GAGTCATTCTCGACTGCTATGTCGACTTTATCATCATCATCTTTATAATCACGTTTGATATCCACTTTGGT). The Donor HCDR3 Swap scFv gene was assembled by overlap-extension based on the $(Gly_4\text{-}Ser)_3$ linker sequence homology between the VH and VL PCR products.

Quantitative real-time PCR

RNA from CLL donor MNCs were converted into cDNA as described in Materials and Methods. Quantitative real-time PCR was done using the Customized TaqMan® Gene Expression Assay (Invitrogen) on a LightCycler® 480 Instrument II (Roche). The sequences for the upstream IGHV1 allele-specific primer, downstream

IGHJ4 allele-specific primer, and the TaqMan probe complementary to the hypervariable V–N–D region of the CLL donor HCDR3 are as follows: upstream primer (GCTGAGCAGCCTGAGATCTG), downstream primer (GGCCCCAGTAGTCAAAGTAGTAC), and probe (CTGTGCGAGAGTTACAGTCAAG). Two independent quantitative real-time PCR reactions with duplicate samples were performed, and the value of the target gene was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The fold change in the CLL donor HCDR3 transcript relative to the GAPDH endogenous control was determined by the following formulas:

Fold Change = $2^{-\Delta(\Delta CT)}$

$\Delta C_T = C_{T,\ \text{CLL donor HCDR3}} - C_{T,\ \text{GAPDH}}$

$\Delta(\Delta C_T) = \Delta C_{T,\ \text{stimulated}} - \Delta C_{T,\ \text{control}}$

Supplementary Figures and Legends

Figure S1

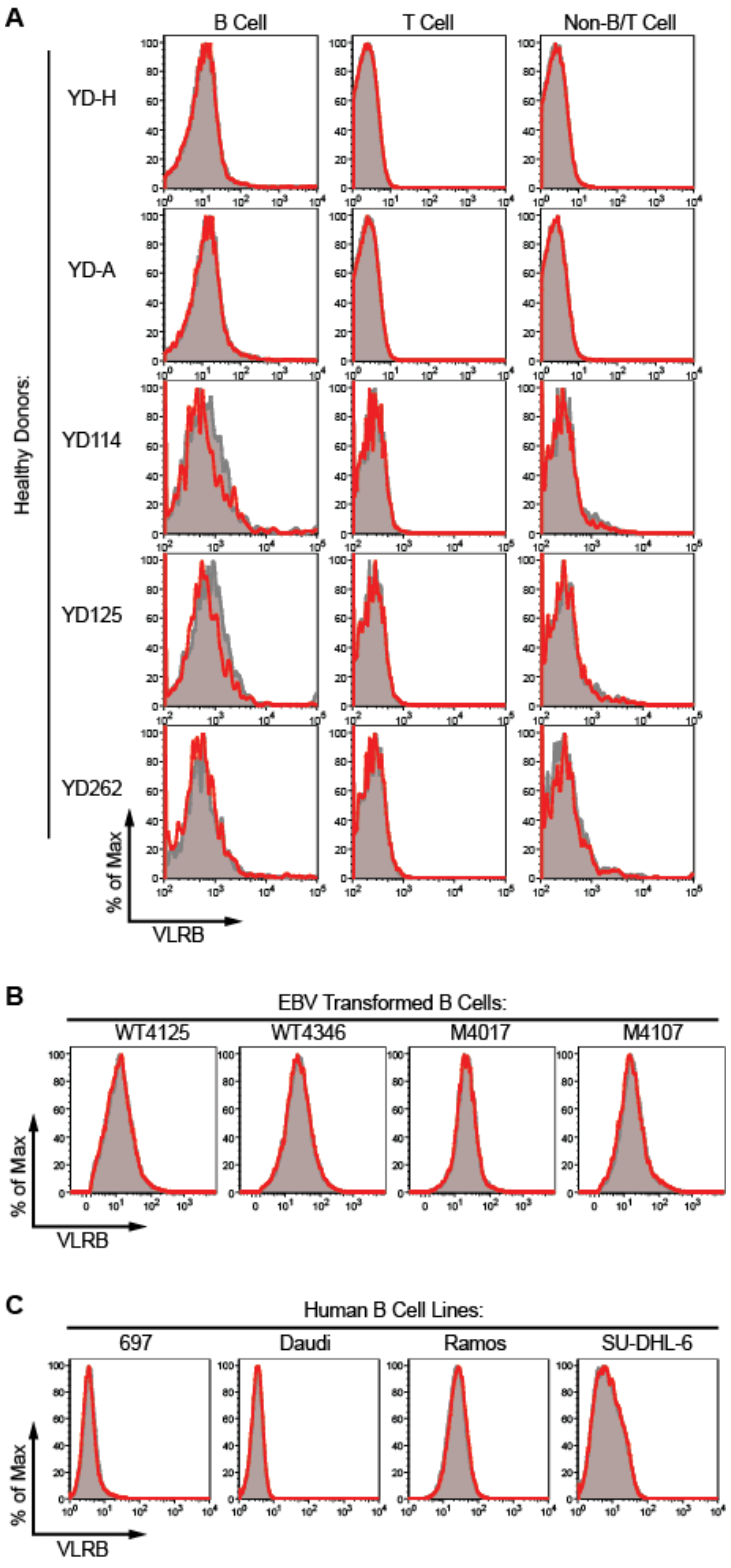

Figure S1: Flow cytometric analysis of monoclonal VLR39 reactivity with different cell types and cell lines. VLR39 binding (red line) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). Histogram of VLR39 binding to (A) five different healthy donor lymphocytes gated on B cells ($CD3^-/CD19^+$), T cells ($CD3^+/CD19^-$), and non-B/T cells ($CD3^-/CD19^-$), (B) four EBV transformed B cells, and (C) four different human B cell leukemia/lymphoma lines, including pre-B cell leukemia (697), Burkitt's Lymphoma (Daudi, Ramos), and diffuse large B cell lymphoma (SU-DHL-6).

Table S1. CLL *VH* gene families and HCDR3 sequences

CLL ID	IGHV Gene	IGHD Gene	IGHJ Gene	HCDR3 amino acid sequence
Donor	IGHV1-69*01	IGHD3-3*01	IGHJ4*02	ARVTVKYYDFWGYYFDY
1153	IGHV1-69*01	IGHD3-3*01	IGHJ4*02	DDSYYDFWSGWYY
1012	IGHV1-69*01	IGHD3-3*01	IGHJ6*03	ARVEIFGVVGLSYYYYYMDV
1640	IGHV1-69*01	IGHD3-3*01	IGHJ6*03	ARGAIFGVVIIPVTPFYMDV
859	IGHV3-09*01	IGHD3-3*01	IGHJ4*02	AKDASSNYDFWSGYYDY
1371	IGHV4-b*02	IGHD3-3*01	IGHJ4*02	ARVMEKYYDFWSGYYYFD
1324	IGHV1-69*01	IGHD3-10*01	IGHJ6*03	AREASYGSGSYYQQYYYYYYMDV
1333	IGHV1-69*01	IGHD3-10*01	IGHJ6*02	AVGVLWFGELLFSYYYYYGMD
352	IGHV1-69*05	IGHD3-3*01	IGHJ6*02	AGRLIFGVVITAGGDYGMDV
1301	IGHV4-31*03	IGHD3-3*01	IGHJ3*02	ARAPIGSTIFGVVIIRFAFDI
276	IGHV1-2*02	IGHD2-21*02	IGHJ4*02	ARTQIGDCGGDCYPFDY
336	IGHV1-3*01	IGHD6-19*01	IGHJ4*02	AREQWLVLSYFDY
606	IGHV1-2*04	IGHD3-10*01	IGHJ4*02	ARDLRYSYGSGSTPFLDS
758	IGHV1-18*01	IGHD3-16*01	IGHJ4*02	ARKSWVGAYYFDY
854	IGHV1-3*01	IGHD2-2*01	IGHJ4*02	VSHYCTSSTCDQMY
1222	IGHV1-2*02	IGHD6-19*01	IGHJ4*02	AREQWLASPNLDY
1271	IGHV4-34*02	IGHD6-6*01	IGHJ4*02	ARGRWSPKFVL
1299	IGHV3-23*01/IGHV3-23*02	IGHD2-2*01	IGHJ4*03	AKGLVIGLPDV
1330	IGHV3-7*03	IGHD2-8*01	IGHJ4*02	ARSSRDGTNDYDGEYRYFDY
1240	IGHV3-33*01/IGHV3-33*06	IGHD3-9*01	IGHJ2*01	ATRPQLNYDILTGYYIGGGYFDL
1294	IGHV3-21*01	IGHD7-27*01	IGHJ6*03	ARDPYRGLYGMFYFYYMDV
1317	IGHV4-59*01	IGHD2-21*02	IGHJ3*02	ARNPYCGGDCYSDAFDI
1319	IGHV4-31*03	IGHD2-2*02	IGHJ6*02	ARDLYGWTYCSSTSCYRYYGMDV
1326	IGHV3-53*04	IGHD5-12*01	IGHJ6*02	ARDRVDIVATTTYYYYYYGMDV
1344	IGHV1-69*02	IGHD2-21*02	IGHJ5*02	ARSTNLDYFFAAVTGNWFDP
1358	IGHV4-34*02	IGHD2-8*01	IGHJ6*02	TRAMDYYYGMDV
1397	IGHV1-69*06	IGHD3-22*01	IGHJ6*02	ATPPRGTYDSSGYYYGGLDNYYGMDV

Table S1: CLL VH gene families and HCDR3 sequences. The *IGHV*, *IGHD*, and *IGHJ* genes and the HCDR3 amino acid sequences expressed in the leukemic cells of 26 CLL patients tested for VLR39 reactivity are shown. Shared VH gene family usage between CLL donor and other CLL patients are highlighted in yellow (*IGHV*), blue (*IGHD*), and green (*IGHJ*). CLL VH gene family determination and all sequences were provided by R. Catera and N. Chiorazzi.

Figure S2

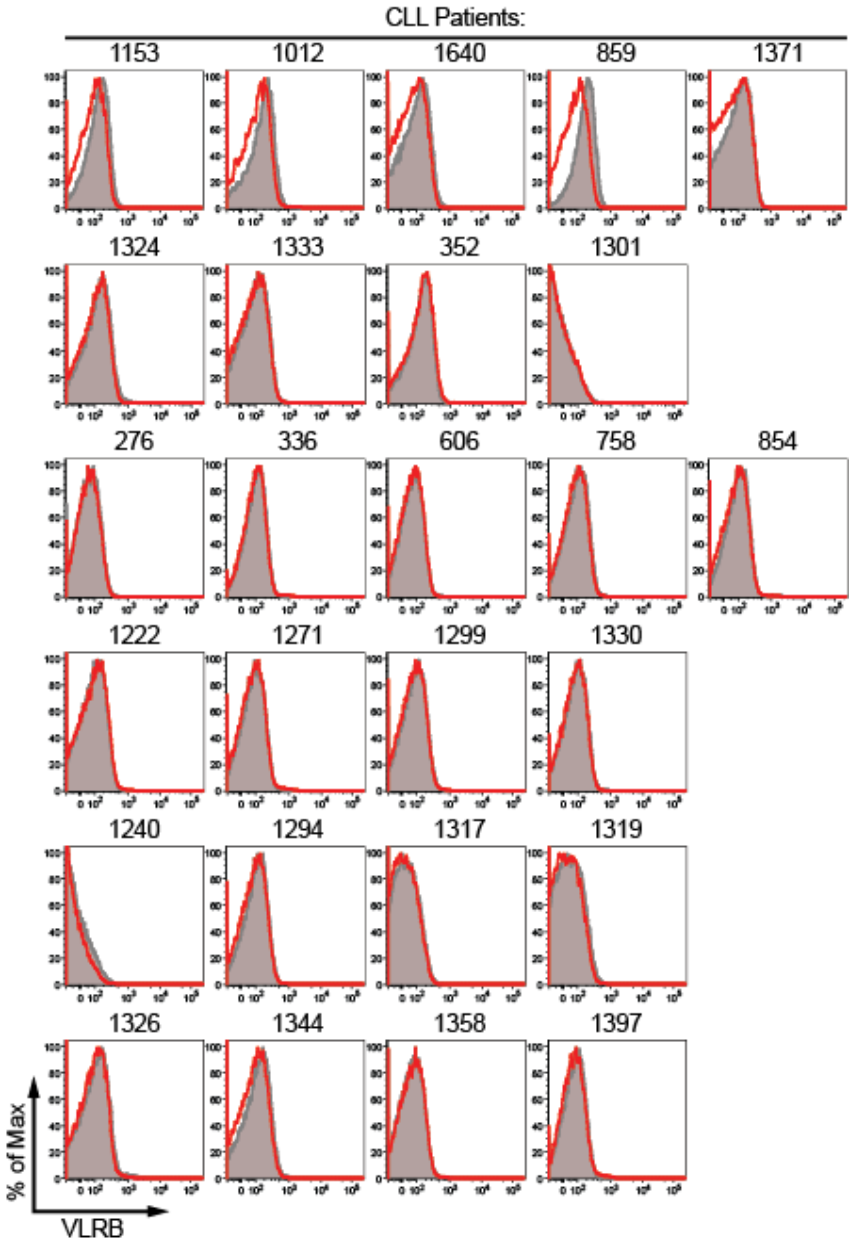

Figure S2: Flow cytometric analysis of monoclonal VLR39 reactivity with CLL cells with different *VH* gene sets. VLR39 binding (red line) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). Histogram of VLR39 binding to 26 CLL patients with known *VH* gene sequences (gated on $CD5^{+}/CD19^{+}$ lymphocytes).

Figure S3

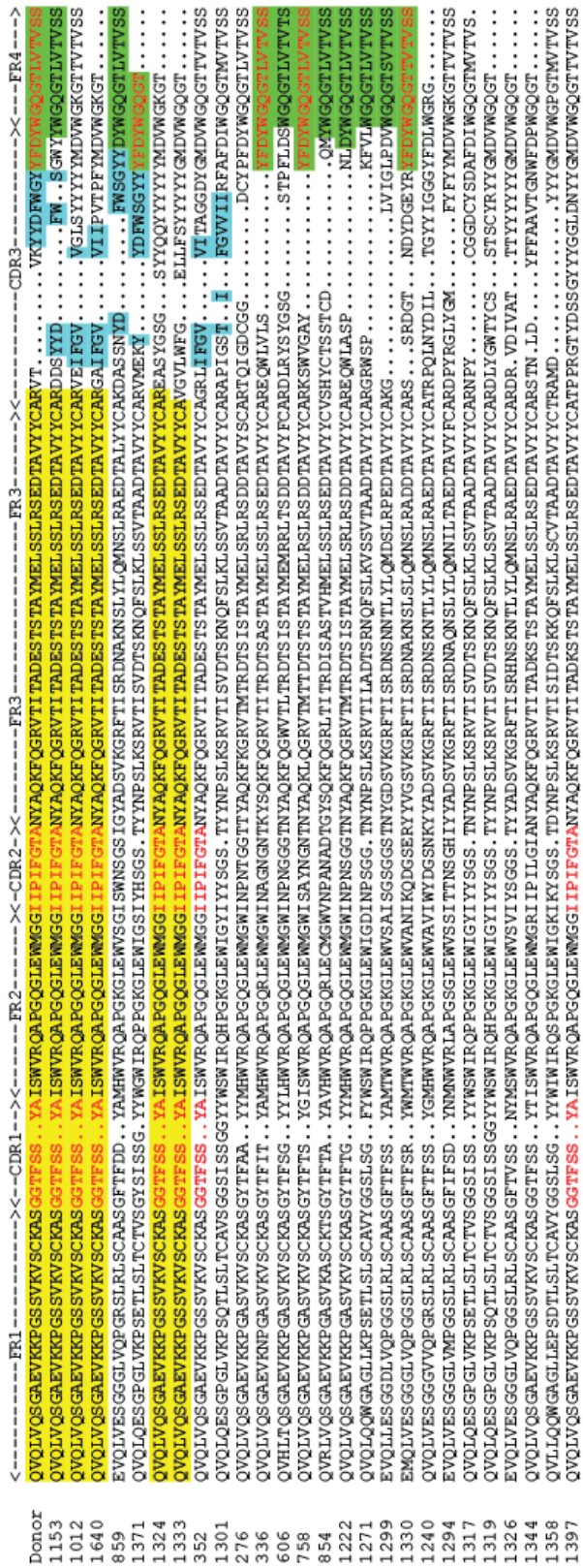

Figure S3: CLL *VH* gene sequence analysis. Sequence alignment between the IGVH-D-J gene rearrangements of CLL donor and those of the 26 CLL patients tested for VLR39 reactivity by flow cytometry are shown. Shared *VH* gene family usage between CLL donor and other CLL patients are highlighted in yellow (*IGVH*), blue (*IGHD*), and green (*IGHJ*). Identical CDR1, CDR2, and IGHJ amino acid sequences are shown in red. All sequences were provided by R. Catera and N. Chiorazzi.

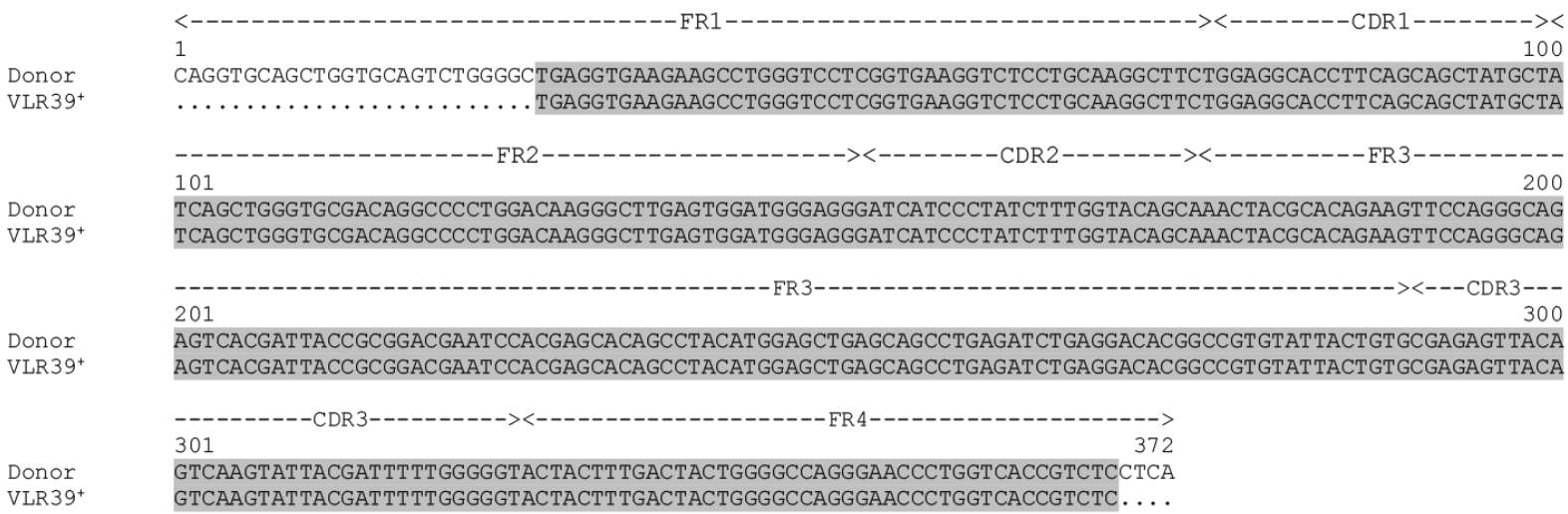
<----------FR1----------><--------CDR1--------><
1 100
Donor CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTA
VLR39+ .....TGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTA
----------FR2----------><--------CDR2--------><----------FR3----------
101 200
Donor TCAGCTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAGCAAACTACGCACAGAAGTTCCAGGGCAG
VLR39+ TCAGCTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAGCAAACTACGCACAGAAGTTCCAGGGCAG
----------FR3----------><---CDR3---
201 300
Donor AGTCACGATTACCGCGGACGAATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGAGTTACA
VLR39+ AGTCACGATTACCGCGGACGAATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGAGTTACA
----------CDR3----------><----------FR4---------->
301 372
Donor GTCAAGTATTACGATTTTTGGGGGTACTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
VLR39+ GTCAAGTATTACGATTTTTGGGGGTACTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTC....

Figure S4

Figure S4: IGHV-D-J sequence alignment of VLR39 sorted cells. Sequence alignment between the IGHV-D-J gene rearrangement of CLL donor and those of the $VLR39^{+}/CD5^{hi}$ cells sorted from the CLL donor B cells at 58 months after treatment. No significant alignment was possible for the $VLR39^{-}/CD5^{lo}$ cells. Nucleotide identity to the original CLL donor IGHV-D-J sequencing results is highlighted in gray.

Table S2. Detection of CLL recurrence by quantitative real-time PCR

Months After End of CFAR Treatment	Donor CLL HCDR3 (C_T)	GAPDH (C_T)	ΔC_T	$\Delta(\Delta C_T)$	Fold Change	CLL HCDR3 Detection
38	not detectable	18.7 ± 0.1	0.00	0	0	negative
51	37.48	25.4 ± 0.4	12.10	2.02	0.25	weak positive
56	26.4 ± 0.1	18.4 ± 0.2	8.02	-2.02	4.05	positive

Table S2: Detection of CLL recurrence by quantitative real-time PCR.

Cryopreserved MNCs from the CLL donor after treatment were analyzed by qPCR for the presence of CLL donor HCDR3 transcript. Each time point is calculated as months after end of CFAR treatment regimen. The C_T , ΔC_T, and $\Delta(\Delta C_T)$ of the qPCR reaction using the CLL donor HCDR3-specific probe and GAPDH probes were used to calculate the fold change in the CLL donor HCDR3 transcript relative to the GAPDH endogenous control. Data generated by X-J. Yan.

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Chapter 3

Recognition of a mouse BCL1 leukemia BCR idiotope by a lamprey antibody.

A version of this chapter has been prepared for submission to *Monoclonal Antibodies in Immunodiagnosis and Immunotherapy* as: Nakahara, H., Herrin, B.R., Cooper M.D. "Recognition of a mouse BCL1 leukemia BCR idiotope by a lamprey antibody."

Abstract

Jawless vertebrates (lamprey and hagfish) possess an unusual adaptive immune system that lacks the conventional Ig/TCR genes used by all other vertebrate species for antigen recognition. Instead, jawless vertebrates use leucine-rich repeat (LRR) sequences to generate three types of variable lymphocyte receptors (VLR): VLRA and VLRB expressed on T-like cells, and VLRB expressed on B-like cells. In response to immunization, VLRB cells proliferate and differentiate into VLRB antibody-secreting plasmacytes. To generate tumor-specific VLRB antibodies, we immunized lampreys with mouse BCL1 leukemia cells to generate recombinant monoclonal VLRB antibodies, which then were screened for specific binding to BCL1 cells and lack of cross-reactivity to other B cells. A tumor-specific clone, VLR-C8, was found to target a B cell receptor idiotope on the complementarity determining region 3 (CDR3) of the heavy and light chain variable region (VH/VL). The ease with which lampreys produce highly specific anti-idiotype antibodies in response to immunization with leukemia cells suggests their usefulness for generating anti-idiotype antibodies that can be used for the analysis of B cell malignancies.

Introduction

Jawless vertebrates (lamprey and hagfish) use variable lymphocyte receptors (VLR) consisting of variable numbers of sequence diverse leucine-rich repeat (LRR) subunits as antigen receptors instead of the Ig-based TCR and BCR used by all jawed vertebrates (1). During VLR gene assembly, the LRR subunits flanking the incomplete germline VLR gene are randomly selected for use as templates to sequentially replace the non-coding intervening sequences in a gene conversion-like process (2,3). In this way VLR assembly can generate a potential VLR repertoire size of greater than 10^{14} unique receptors comparable in size to the mammalian antibody repertoire (4). Allelic exclusion ensures that a single, unique VLR gene is expressed by each lymphocyte (1,4).

Lampreys (5–7) and hagfish (8) have three types of VLR. VLRA and VLRC cells are T-like cells that assemble their receptors in a thymus-equivalent structure in the gills known as thymoids (7,9), whereas VLRB assembly in B-like cells occurs in the typholosole and kidneys. All of the VLRs are expressed as cell surface receptors, but only VLRB cells are capable of secreting multimeric VLRB antibodies as plasma cells after undergoing proliferation and differentiation in response to antigen stimulation (10,11).

Much like other LRR family proteins, the VLR proteins form a crescent-shaped, solenoidal shape (12–15). Antigen contact occurs on the VLR concave surface formed by the parallel β-strands of the variable LRR and, for VLRA and VLRB, an extended hypervariable loop of the C-terminal LRR (13–16). Recombinant, monoclonal VLR proteins have been produced by cloning VLR cDNA from lymphocytes followed by expression in HEK-293T cells (10) or yeast surface display libraries (17); the monoclonal

VLRs can then be screened for antigen-specificity. This approach has yielded highly specific monoclonal VLRB antibodies against a variety of antigens: the BclA protein of *Bacillus anthracis* exosporium (10,16), the H-trisaccharide antigen of human blood group O erythrocytes (13), HEL (14), human CD5 (18), BCR idiotope of a B cell chronic lymphocytic leukemia clone (19), the TFα disaccharide pancarcinoma antigen (15), and others (17,20).

The VLRB antibodies represent a new class of antigen-specific binding molecules that may recognize novel epitopes due to their unique structure which utilizes a rigid LRR β-strand surface and a flexible, extended LRRCT loop for antigen binding (13–16). They have been shown to have exquisite specificity for glycan determinants (13,15,16), specificities that exceed those of conventional antibodies (21) and lectins (22). The absence of antigens common to mammals in jawless vertebrates should also allow VLRB to recognize epitopes that represent an immunologic blind spot for mammalian antibodies, the repertoires of which are biased through germline V(D)J selection and immune tolerance mechanisms.

In a previous study (19), a VLRB library from lamprey immunized with a patient's B cell chronic lymphocytic leukemia (CLL) cells was screened for tumor-specific VLRB clones. We identified a monoclonal VLRB antibody, VLR39, which was specific for the patient CLL clone and proved to target a VH CDR3 idiotope of the BCR. In the present study, we immunized lampreys with mouse BCL1 leukemia cells to generate and characterize tumor-specific VLRB clones that could be developed as tumor-targeting reagents. We identified a monoclonal VLRB antibody, VLR-C8 which also proved to target a BCR idiotope shared between VH CDR3 and VL CDR3, and to be

capable of binding to BCL1 cells as a monomeric VLRB antibody and a dimeric VLR-Fc fusion protein.

Material and Methods

Cells and cell lines

BCL1 cells were obtained from Drs. Jonathan W. Uhr and Ellen S. Vitetta (University of Texas Southwestern Medical Center, Dallas, TX), and maintained by *in vivo* passage in BALB/c mice. The *in vitro* culture adapted strain, BCL1-3B3, was obtained from Drs. Jerry M. Boss and Samuel H. Speck (Emory University, Atlanta, GA). Mouse B cell lines were maintained in RPMI 1640 media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1% MEM non-essential amino acids solution (GIBCO), 0.05 mM 2-mercaptoethanol, and 10% FBS at 37°C in 5% CO_2. HEK-293T cells were maintained in DMEM supplemented with 5% FBS at 37°C in 5% CO_2.

BCL1 mouse leukemia model

BCL1 is a murine IgM/Igλ B cell leukemia of BALB/c origin (23). Transfer of BCL1 cells into normal BALB/c mice induces hepatosplenomegaly and leukemia with death occurring at 2 to 4 months (24). The BCL1 tumor does not survive *in vitro* culture, and is maintained by *in vivo* passage in BALB/c mice. Six-to-eight week old BALB/cAnNHsd mice were obtained from Harlan (Indianapolis, IN) and maintained at Emory University under standard conditions. Two months after intraperitoneal BCL1 tumor injection, the animals were euthanized and the splenocytes were harvested by masceration of whole spleen with frosted glass slides followed by incubation with ACK Lysing Buffer for erythrocyte lysis, and then washing with PBS. The splenocytes were

cryopreserved or directly injected into naïve BALB/c mice for *in vivo* passage. All experiments were approved by the Institutional Animal Care and Use Committee at Emory University.

Antibodies and flow cytometry

The VLRB-specific 8A5 mouse IgG1/κ monoclonal antibody (mAb) was labeled with Alexa Fluor 488 or Alexa Fluor 647 Protein Labeling Kit (Invitrogen), and recognizes 80% of VLRB clones as described previously (19). Unlabeled goat anti-mouse IgM, goat anti-mouse Ig(H+L)-APC, rat anti-mouse antibodies Lambda-PE and CD19-APC were obtained from Southern Biotech. Propidium iodide and 7-AAD were obtained from BD Biosciences.

Lamprey immunization, VLRB cDNA library screening, purification of monoclonal VLRB antibody

Lamprey larvae were maintained as previously described (4) and immunized with three biweekly intracoelomic injections of $1x10^7$ splenocytes from BCL1-tumor bearing mice. Two weeks after the third immunization, the lampreys were sacrificed and the plasma and buffy coat were collected. BCL1-reactive VLRB production was assessed by incubating BCL1 splenocytes with the collected lamprey plasma, then with Alexa Fluor 647 dye-conjugated 8A5 anti-VLRB mAb, with cell washing between each incubation step before flow cytometric analysis. Of the 6 immunized animals, the buffy coat leukocytes from the lamprey with the highest titer of BCL1-reactive VLRB antibodies was used to construct a VLRB cDNA library which was expressed as recombinant

monoclonal VLRB antibodies as described previously (10,19). To assess the BCL1 specificity of the monoclonal VLRB antibodies, splenocytes from BCL1 tumor-bearing mice and healthy control mice were sequentially incubated with VLRB transfectant supernatants, followed by Alexa Fluor 647 dye-conjugated 8A5 anti-VLRB mAb, before blocking with 5% mouse serum and staining for Igλ chain and CD19. Cells were washed in PBS between each incubation step and propidium iodide added before flow cytometric analysis of the cells. After obtaining the recombinant monoclonal VLRB antibody of interest, they were purified using its N-terminal hexahistidine-tag as described previously (19). All experiments were approved by the Institutional Animal Care and Use Committee at Emory University.

Surface Ig modulation

BCL1-3B3 cells were incubated with 2.5 µg/ml of unlabeled goat anti-mouse IgM without sodium azide(Southern Biotech) in culture media at 37°C in 5% CO_2 for 30, 10, or untreated before sequentially incubated with VLRB transfectant supernatants followed by Alexa Fluor 647 dye-conjugated 8A5 anti-VLRB mAb, or PBS followed by goat anti-mouse Ig(H+L)-APC. Cells were washed in PBS between each incubation step before flow cytometric analysis of the cells.

Cloning, expression, and sequence analysis of mouse BCL1 BCR

The BCL1 BCR was cloned as a single-chain variable fragment (scFv) as previously described (19). Briefly, RNA from BCL1-3B3 cells were converted into cDNA, and the VH and VL regions were amplified using an overlap-extension PCR

strategy with mouse V gene primer sequences (25) adapted for use in a published protocol (22). Primer and PCR cycle profile are detailed in Supplementary Materials and Methods. The scFv plasmid was expressed on the surface of HEK-293T cells by PEI transfection and harvested as described previously (10,19). After confirming VLR-C8 reactivity with the BCL1 scFv, four hybrid scFv mutants were constructed by swapping the VH CDR3 or VL CDR3 sequence between the BCL1 and CLL scFv as described in Supplementary Materials and Methods.

Analysis of VLR-C8 idiotope specificity for BCL1 BCR

For flow cytometric analysis, the scFv transfected 293T were sequentially incubated with 0.04 mg/ml purified VLR protein, followed by Alexa Fluor 647-conjugated 8A5 anti-VLRB mAb and Anti-Myc Tag, clone 4A6, Alexa Fluor 488 conjugate (Millipore) and 7-AAD to verify cell surface expression of transfected scFv; cells were washed in PBS between each incubation step.

VLRB monomer and dimeric VLR-Fc fusion protein

For the VLRB monomer, the VLRB sequence from the LRRNT to the Thr/Pro-rich stalk sequence up to the GYVATTT was PCR amplified using a 5’ primer containing a NheI site, N6H-VBFc_NheI_Fwd (GAGAGCTAGCGCATGTCCCTCGCAGTGTT) and 3’ primer containing a BamHI site, N6H-VBFc_BamHI_Rev (GAGAGGATCCGGTCGTAGCAACGTAGCCTG) with the following cycle profile: 94°C for 5 min, (94°C, 1 min; 52°C, 1 min; and 72°C, 3 min) × 35, 72°C for 7 min. PCR products were purified by QIAquick Gel Extraction Kit (Qiagen), digested with NheI and

BamHI (New England Biolabs), and cloned into a pIRES-puro-2 vector (Clontech) modified to introduce a hexahistidine-tag between the lamprey VLRB signal peptide and LRRNT (19) using T4 DNA ligase (New England Biolabs). VLRB monomer expression plasmid was purified from transformed NovaBlue competent cells (Novagen) with the QIAprep Spin Miniprep Kit (Qiagen). The purified plasmid was transfected into HEK-293T cell lines, and stable transfectants were selected with 2 μg/ml puromycin (Sigma) before expansion in CELLine AD 1000 Flasks (Argos Technologies). The bioreactor supernatants were harvested every 7 days, and the recombinant VLRB protein was purified from supernatant via the hexahistidine tag using Ni-NTA agarose (Qiagen) as described previously (19). For the dimeric VLR-Fc fusion protein, the same PCR fragment was cloned into the hexahistidine-tagged pIRES-puro-2 vector (Clontech) modified to introduce a mouse IgG2a-Fc sequence including the hinge region, right after the BamHI cloning site and then purified as above.

BCL1-3B3 cells were sequentially incubated with VLRB monomer, VLR-Fc fusion protein, or VLRB wildtype decamer as supernatant or purified protein, followed by Alexa Fluor 647 dye-conjugated 8A5 anti-VLRB mAb and 7-AAD. Cells were washed in PBS between each incubation step before flow cytometric analysis of the cells.

Results and Discussion

Identification of a BCL1-specific monoclonal VLRB antibody

Individual clones selected randomly from the BCL1-immunized *VLRB* cDNA library were expressed by transfection into HEK-293T cells, and the transfectant supernatants were analyzed for binding to viable BCL1 cells by immunofluorescence analysis. Since the ratio of Igκ:Igλ B cells is greater than 10:1 in healthy mice (26), Igλ was used as a surrogate marker for expansion of IgM/Igλ BCL1 cells (Figure 1A). One monoclonal VLRB antibody, VLR-C8, reacted with the BCL1 cells phenotyped as CD19+/Igλ+ splenocytes from BCL1 tumor-bearing mice, but had minimal reactivity with splenocytes from healthy mice (Figure 1B). Despite the minimal background staining observed for splenocyte preparations, VLR-C8 specificity for the BCL1 cell line was validated by the complete absence of staining against other mouse B cell lines (Figure 1C).

VLR-C8 reactivity after down-modulation of surface Ig

Based on our previous identification of a tumor-specific VLRB anti-idiotypic antibody (19), we examined the possibility that VLR-C8 was also an anti-idiotypic antibody. The ligation of cell surface Ig by anti-Ig antibody induces its redistribution and pinocytosis (27). VLR-C8 reactivity against the BCL1-3B3 cells was found to be reduced by BCR modulation after incubation with anti-IgM antibodies (Fig 2, top panel); the down-modulation of surface Ig was confirmed by cell surface Ig staining with anti-Ig(H+L) (Fig 2, bottom panel). The VLR-C8 staining intensity was reduced

proportionately with the reduction of surface Ig staining intensity, thereby indicating that VLR-C8 binds to the surface Ig or an associated component of the BCR.

VLR-C8 recognizes the VH/VL CDR3 sequence of the donor CLL

To examine the possibility that VLR-C8 recognizes an Ig idiotope of the BCL1 tumor cells, the VH and VL regions were sequenced and expressed on transfected cells as a single-chain variable fragment (scFv) with a myc-tag. The transfected cells were then evaluated for anti-myc and VLR-C8 binding by immunofluorescence flow cytometry analysis. VLR-C8 was found to react with BCL1 Ig, but not with a control scFv from a CLL donor (Figure 4, left column). To test whether VLR-C8 was recognizing a BCR CDR3 idiotope, the VH/VL CDR3 of the BCL1 Ig and the CLL donor Ig were swapped to construct four hybrid scFv mutants (Figure 3); there were expressed as membrane-bound scFv on the transfected cells and VLR-C8 reactivity was evaluated by immunofluorescence flow cytometry analysis (Figure 4, middle and right column) as in our previous CLL patient study (19). The permutations that were evaluated were as follows:

A. Hybrid scFv A replaces the VH CDR3 signature sequence of the BCL1 Ig with the VH CDR3 of CLL donor Ig, but retains the BCL1 VL.
B. Hybrid scFv B replaces the VH CDR3 signature sequence of the CLL donor Ig with the VH CDR3 of BCL1 Ig, but retains the CLL donor VL.
C. Hybrid scFv C replaces the VL CDR3 signature sequence of the BCL1 Ig with the VL CDR3 of CLL donor Ig, but retains the BCL1 VH.

D. Hybrid scFv D replaces the VL CDR3 signature sequence of the CLL donor Ig with the VL CDR3 of BCL1 Ig, but retains the CLL donor VH.

Of the hybrid scFv mutants, VLR-C8 staining intensity was highest for the "C" mutant product, which retains a fully intact BCL1 VH and lacks the BCL1 VL CDR3. However, placing the BCL1 VH CDR3 sequence within the FR3/4 of the CLL VH did not confer VLR-C8 binding in "B". This suggests that the YYGN amino acid sequence of the BCL1 VH CDR3 alone is insufficient for optimal VLR-C8 binding.

The missing BCL1 VL CDR3 sequence in "C" may explain its lower staining intensity when compared to the wildtype BCL1 Ig with its fully intact VH/VL. This result can be interpreted to indicate that the ALWYINHFI amino acid sequence of the BCL1 VL CDR3 is necessary for optimal VLR-C8 binding; this possibility is further bolstered by the slight VLR-C8 reactivity observed in "A", which retains a fully intact BCL1 VL, but is missing the BCL1 VH CDR3. As with the BCL1 VH CDR3 sequence in "B", placing the BCL1 VL CDR3 sequence by itself within the FR3/4 of the CLL VL did not confer VLR-C8 binding in "D".

Altogether, the VLR-C8 reactivity observed for the scFv mutants with intact BCL1 VH or VL ("C" and "A" respectively) suggests that the VLR-C8 epitope is comprised of both the VH/VL CDR3 of the BCL1 Ig, albeit with a stronger contribution from the VH CDR3 than from the VL CDR3. The lack of VLR-C8 reactivity in the scFv mutants containing BCL1 VH/VL CDR3 sequence in isolation ("B" and "D" respectively) could be due to a VH/VL CDR3 conformation alteration that is unfavorable for VLR-C8 binding that is enforced by the flanking CLL VH/VL FR3/4.

VLR-C8 monmeric and dimeric forms bind to BCL1 cells

VLRB antibodies are secreted as 320 to 400 kDa multimers composed of identical subunits that form disulfide-linked dimers joined to each other at the carboxyl-terminus to form octamers and decamers (10,11). The large size of wildtype VLRB decamers make them unsuitable for many *in vivo* applications, due to limited tissue penetration, slow clearance, and immunogenicity. The smaller sized VLRB monomers (15 to 25 kDa) would be expected to be better suited for *in vivo* applications. However, the affinity of individual VLRB monomers is often in the low micromolar range and therefore may be insufficient for successful antigen-binding (14,28). For immunotherapy purposes, a dimeric VLR-Fc fusion protein could increase the antigen-binding avidity and would also allow for Fc-Fc receptor interactions (29,30).

To evaluate whether the VLR-C8 antibody has sufficient affinity for *in vivo* tumor-targeting, monomeric and dimeric VLR-Fc fusion proteins were tested for binding to BCL1 cells by immunofluorescence flow cytometry analysis (Figure 5). Both the VLR-C8 monomer and VLR-C8-Fc dimer displayed a nearly 2 log shift in staining intensity over the negative control VLR4, a level of reactivity which is comparable to that observed for the VLR-C8 wildtype decamer. As would be expected from the number of VLRB subunits available for avidity interactions, the highest MFI was observed with the decamer, followed by the dimer, and then the monomer.

Although the original goal of screening VLRB antibodies from leukemia-immunized lampreys was in search of potentially novel epitopes, it is not surprising in retrospect that screening for tumor-specificity yielded results comparable to those obtained in our screening for idiotopes on the malignant B cells from a CLL patient (19).

VLR-C8 is thus the second such anti-idiotypic VLRB to be identified, and the location of the epitope on the Ig VH/VL CDR3 suggests that the BCR iditope may be favorably immunogenic in lampreys. This possibility was not anticipated, although the absence of BCRs and TCRs in jawless vertebrates (31,32) could bias the VLRB repertoire towards neo-antigens formed by V(D)J recombination as well as the rest of the Ig molecule. Solving the structure of a VLRB-Ig complex for comparison with known Ig idiotype-anti-idiotype structures may yield further insight (33–38). The tumor-specificity and affinity of individual VLR-C8 monomers allows the future evaluation of tumor targeting *in vivo* using the mouse BCL1 leukemia model. In summary, our data indicates the feasibility of using idiotypic antibodies for use in the diagnosis and monitoring of B cell malignancies.

Figures and Legends

Figure 1

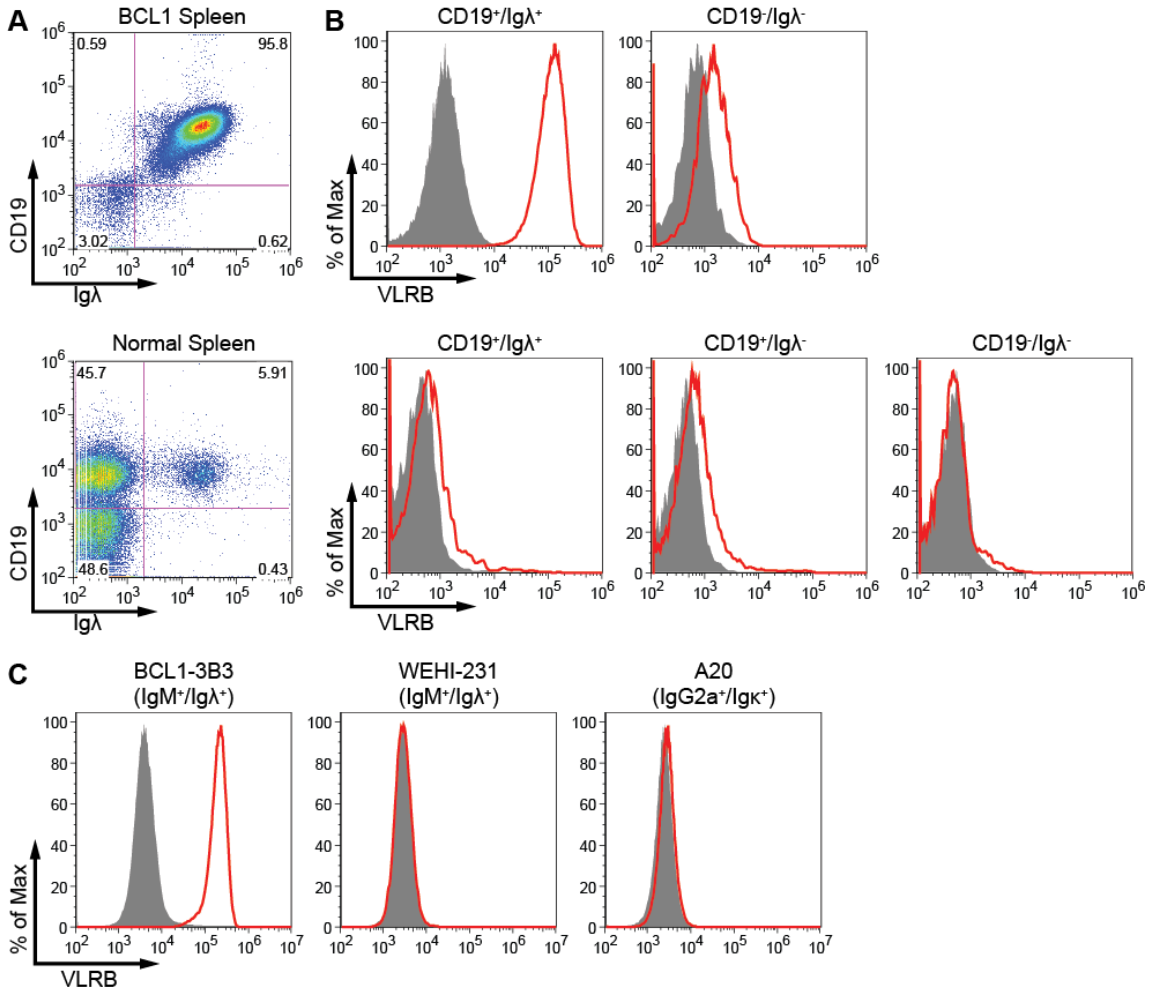

Figure 1: Flow cytometric analysis of monoclonal VLR-C8 reactivity. VLR-C8 binding (red) was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading). (A) Anti-CD19 and anti-Igλ chain staining of splenocytes from BCL1 injected (top panel) or healthy control mice (bottom panel). (B) Histogram of VLR-C8 binding to splenocytes from BCL1-injected mice were gated on BCL1 cells ($CD19^+/Ig\lambda^+$), and non-B/T cells ($CD19^-$/ $Ig\lambda^-$) (top row). Histogram of VLR-C8 binding to normal splenocytes gated on $Ig\lambda^+$ B cells ($CD19^+/Ig\lambda^+$), $Ig\kappa^+$ B cells ($CD19^+/Ig\lambda^-$) and

non-B/T cells ($CD19^-$/ $Ig\lambda^-$) (bottom row). (C) Histogram of VLR-C8 binding to the indicated mouse B cell lines.

Figure 2

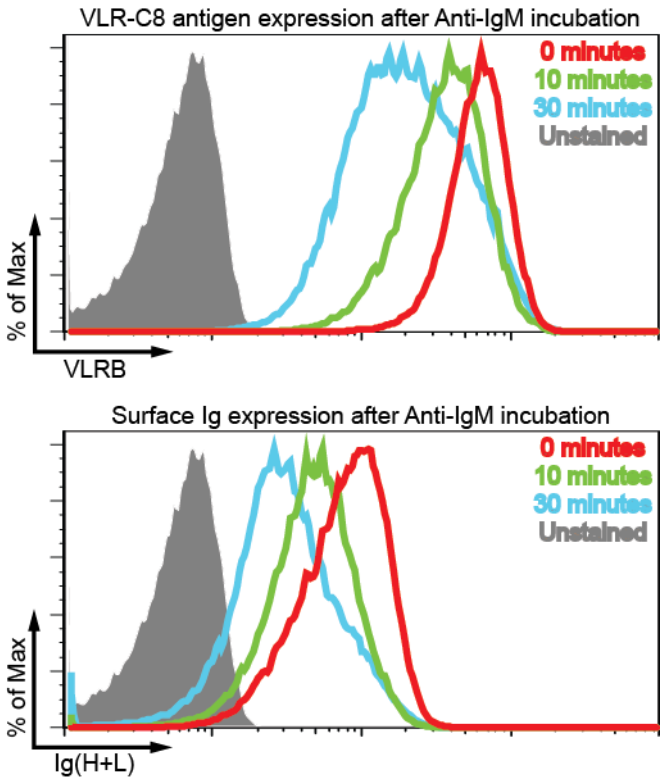

Figure 2: Flow cytometric analysis of VLR-C8 binding to BCL1-3B3 cells after surface Ig modulation. VLR-C8 binding to BCL1-3B3 (top panel) was compared with that of polyclonal goat anti-mouse Ig(H+L) (bottom panel) following incubation of BCL1-3B3 cells with anti-mouse IgM antibody for the indicated amount of time. Histogram of VLR-C8 binding (upper panel) or polyclonal goat anti-mouse Ig(H+L) chain antibody (lower panel) to BCL1-3B3 that were either untreated (red), incubated with anti-mouse IgM antibody for 10 minutes (green), or for 30 minutes (blue). Unstained controls are shown in gray.

Figure 3

A

```
                <----------FR1----------><-CDR1-><------FR2------><-CDR2-><-----------------FR3-----------------><-----CDR3------><---FR4----
BCL1 IgH        QVQLQQSGPEVVRPGVSVKISCKGSGYTFTDYVMHWVKQTHAKSLEWIGVISTYNGNTYYNQKFKGKATMTVDKSSSTAYMELARLTSEDSAIYYCAR-------YYGNYFDYWGQGTTLTVSS
CLL IgH         QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARVTVKYYDFWGYYFDYWGQGTLVTVSS
                <----------FR1----------><--CDR1--><------FR2------>CD2<---------------FR3----------------><--CDR3--><---FR4----
BCL1 IgL        DAVVTQE-SALTTSPGETVTLTCRSSTGAVTTSDYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALW-YINHFIFGSGTKLTVLG
CLL IgL         DIQMTQSPSSLSASVGDRVTITCQASQD---ISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNLPVFTFGPGTKVDIK
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B

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                                <----------------FR3-----------------><-----HCDR3-----><---FR4----
BCL1 IgH containing CLL HCDR3    YYNQKFKGKATMTVDKSSSTAYMELARLTSEDSAIYYCARVTVKYYDFWGYYFDYWGQGTTLTVSS
CLL IgH containing BCL1 HCDR3   NYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCAR-------YYGNYFDYWGQGTLVTVSS
                                <----------------FR3-----------------><-LCDR3--><---FR4----
BCL1 IgL containing CLL LCDR3   NRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCQQYDNLPVFTFGSGTKLTVLG
CLL IgL containing BCL1 LCDR3   NLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCALW-YINHFIFGPGTKVDIK
```

C

Name of scFv	Heavy chain	Light chain
BCL1 scFv	BCL1 IgH	BCL1 IgL
CLL scFv	CLL IgH	CLL IgL
Hybrid scFv A	BCL1 IgH containing CLL HCDR3	BCL1 IgL
Hybrid scFv B	CLL IgH containing BCL1 HCDR3	CLL IgL
Hybrid scFv C	BCL1 IgH	BCL1 IgL containing CLL LCDR3
Hybrid scFv D	CLL IgH	CLL IgL containing BCL1 LCDR3

Figure 3: Amino acid sequence alignment of Ig heavy and light chains comprising the hybrid scFv mutants. (A) Amino acid sequence alignment of Ig heavy and light chains of the BCL1 and CLL clones. The HCDR3 signature sequences of mouse BCL1 (red text) and the HCDR3 signature sequences of human CLL (blue text) are highlighted in gray. The LCDR3 signature sequences of mouse BCL1 (red text) and the LCDR3 signature sequences of human CLL (blue text) are underlined. (B) The swapped portion of the CDR3 signature sequence used to construct the hybrid scFv mutants are shown in gray highlight. (C) The name of the 6 scFv expression constructs, and the Ig heavy and light chain combination comprising them are listed.

Figure 4

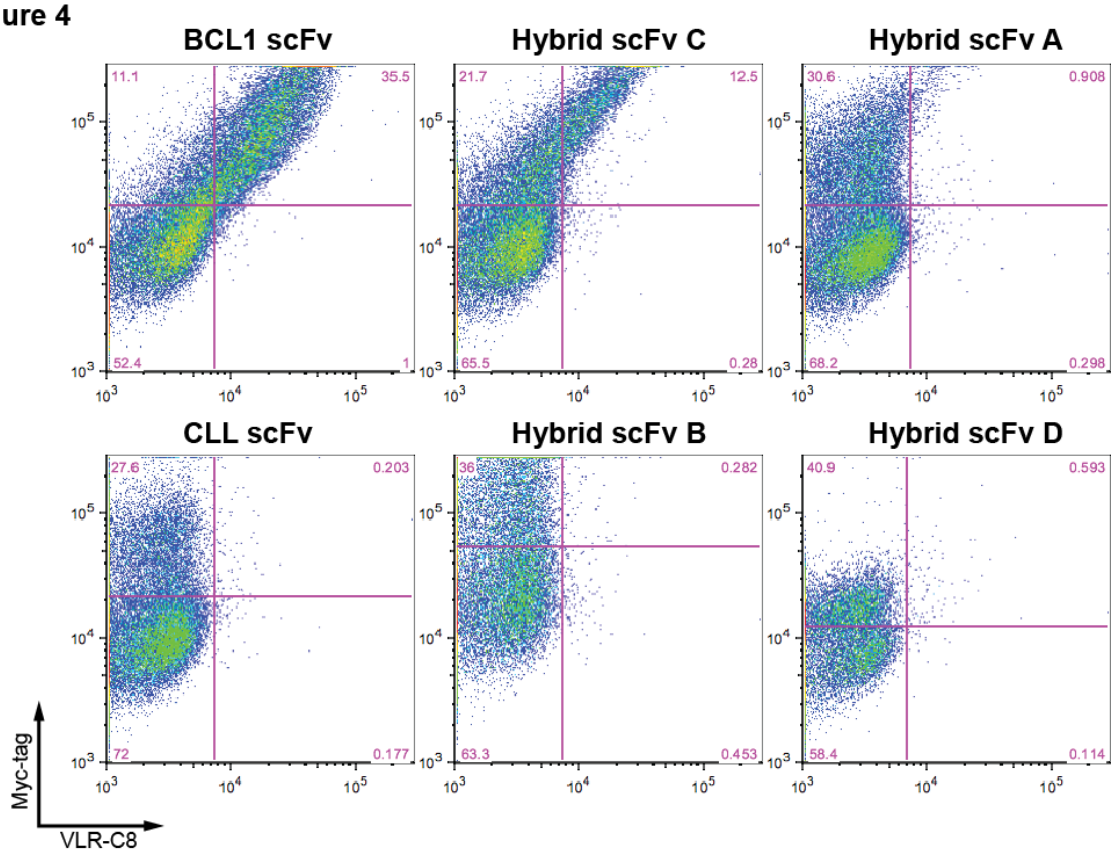

Figure 4: VLR-C8 binding to BCL1, CLL, and hybrid scFv. Flow cytometric analysis of VLR-C8 binding to HEK-293T cells transfected with BCL1 scFv, CLL scFv, or the four hybrid scFv mutants. Myc-tag was co-stained to measure scFv surface expression.

Figure 5

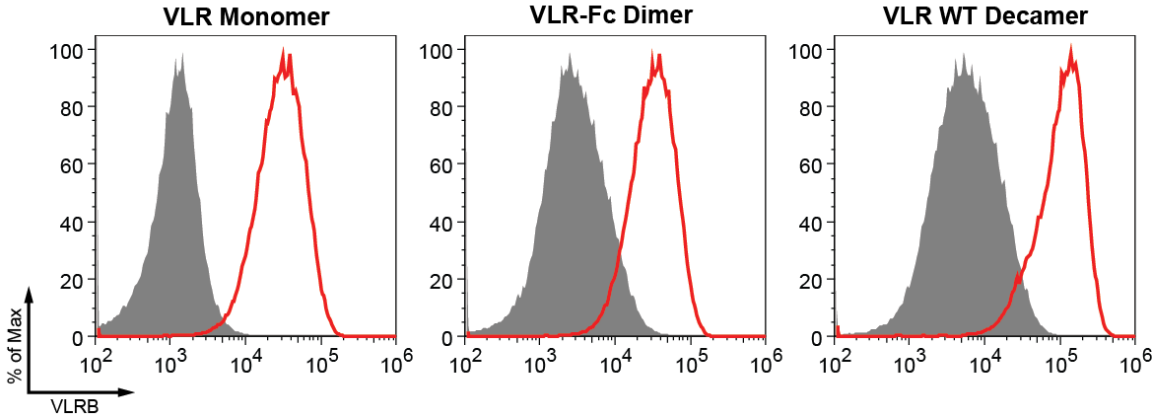

Figure 5: Flow cytometric analysis of VLR-C8 expressed as a monomer and Fc-fusion protein. VLR-C8 binding (red) to BCL1-3B3 was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity (gray shading) either as monomer, VLR-Fc dimer, or VLR wildtype decamer.

Supplementary Materials and Methods

Cloning of BCL1 BCR as scFv

Briefly, for the first round of PCR, the VH region was amplified using a 5' primer containing a XmaI site, BCL1_VH_FR1_XmaI_Fwd (GTCGACCTGCAGACAGAGTTACCCGGGGCCCAACCAGCGATGGCCCAGGTTCAGCTGCAGCAGTC) and 3' primer containing a $(Gly_4\text{-}Ser)_3$ linker sequence, BCL1_VH_FR4_scFv_Rev (CCGCCGGATCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACTGTGAGAGTGG) with the following cycle profile: 94°C for 5 min, (94°C, 1 min; 52°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min. For VL region amplification, a 5' primer containing a $(Gly_4\text{-}Ser)_3$ linker sequence, BCL1_VL_scFv_Fwd (GGAGGCGGTTCAGGCGGAGGTGGATCCGGCGGTGGCGGATCGGACGCTGTTGTGACTCAGGA) and 3' primer containing a SalI site and FLAG tag, BCL1_VL_FR4_SalI_Rev (GAGTCATTCTCGACTGCTATGTCGACTTTATCATCATCATCTTTATAATCACGGCCTAGGACAGTCAMCYTGG) were used with the following cycle profile: 94°C for 5 min, (94°C, 1 min; 52°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min. For the second round of PCR, the scFv gene was assembled by overlap-extension based on the $(Gly_4\text{-}Ser)_3$ linker sequence homology between the VH and VL PCR products using the following cycle profile: 94°C for 5 min, (94°C, 1 min; 55°C, 1 min; and 72°C, 3 min) × 6, 72°C for 7 min. In the third round of PCR, the assembled scFv gene was PCR amplified using the outside primers, BCL1_VH_FR1_XmaI_Fwd and

BCL1_VL_FR4_SalI_Rev using the following cycle profile: 94°C for 5 min, (94°C, 1 min; 55°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min. PCR products were purified by QIAquick Gel Extraction Kit (Qiagen), digested with XmaI and SalI (New England Biolabs), and cloned into pDISPLAY vector (Invitrogen) using T4 DNA ligase (New England Biolabs). BCL1 scFv plasmid was purified from transformed NovaBlue competent cells (Novagen) with the QIAprep Spin Miniprep Kit (Qiagen).

Construction of scFv mutants

The four scFv mutants were constructed by amplifying the appropriate VH and VL regions from the BCL1 scFv plasmid and the CLL donor scFv plasmid (19) utilizing overlap-extension PCR as described above, except using different primer sets for the first round of PCR using the following cycle profile: 94°C for 5 min, (94°C, 1 min; 50°C, 1 min; and 72°C, 3 min) × 25, 72°C for 7 min.

Hybrid scFV mutant A: BCL1 scFv 5’ fragment was amplified using a 5’ primer containing a XmaI site, BCL1_VH_FR1_XmaI_Fwd (GTCGACCTGCAGACAGAGTTACCCGGGGCCCAACCAGCGATGGCCCAGGTTCAGCTGCAGCAGTC) and 3’ primer containing the CLL donor VH CDR3 sequence as overhang, BCL1_VH_FR3-CLL655_VH_CDR3_Rev (GTACCCCCAAAAATCGTAATACTTGACTGTAACTCTTGCACAGTAATAGATGGCAGA). The BCL1 scFv 3’ fragment was amplified using a 5’ primer containing the CLL donor VH CDR3 sequence as overhang, CLL655_VH_CDR3-BCL1_VH_FR4_Fwd (GTCGACCTGCAGACAGAGTTACCCGGGGCCCAACCAGCGATGGCCCAGGTT

CAGCTGCAGCAGTC) and 3’ primer containing the terminal end of the BCL1 scFv, BCL1_VL_FR4_SalI_Rev (GAGTCATTCTCGACTGCTATGTCGACTTTATCATCATCATCTTTATAATCACG GCCTAGGACAGTCAMCYTGG). Hybrid scFv mutant A was assembled by overlap-extension based on the CLL donor VH CDR3 sequence homology between the two PCR products.

Hybrid scFV mutant B: CLL donor scFv 5’ fragment was amplified using a 5’ primer containing a XmaI site, CLL655_VH_FR1_XmaI_Fwd (GTCCTCGCAACTGCCCCATCCCGGGGCCCAACCAGCGATGGCCCAGGTGCA GCTGGTGCAGTCTGG) and 3’ primer containing the BCL1 VH CDR3 sequence as overhang, VH_CLL655_FR3-BCL1_CDR3_Rev (GTAGTCAAAGTAGTTACCATAGTATCTCGCACAGTAATACACGGC). The CLL donor 3’ fragment was amplified using a 5’ primer containing the BCL1 donor VH CDR3 sequence as overhang, VH_BCL1_CDR3-655_FR4_Fwd (GCGAGATACTATGGTAACTACTTTGACTACTGGGGCCAGG) and 3’ primer containing the terminal end of the CLL donor scFv, CLL655_VL_FR4_SalI_Rev (GAGTCATTCTCGACTGCTATGTCGACTTTATCATCATCATCTTTATAATCACG TTTGATATCCACTTTGGT). Hybrid scFv mutant B was assembled by overlap-extension based on the BCL1 VH CDR3 sequence homology between the two PCR products.

Hybrid scFV mutant C: The BCL1 scFv 5’ fragment was amplified using a 5’ primer containing a XmaI site, BCL1_VH_FR1_XmaI_Fwd (GTCGACCTGCAGACAGAGTTACCCGGGGCCCAACCAGCGATGGCCCAGGTT

CAGCTGCAGCAGTC) and 3’ primer containing the CLL donor VL CDR3 sequence as overhang, VL_BCL1_FR3-CLL655_CDR3_Rev (AGTGAATACAGGGAGATTATCATACTGTTGACAGAAATATATTGCCTCATCCTCA). The BCL1 scFv 5’ fragment was amplified using a 5’ primer containing the CLL donor VL CDR3 sequence as overhang, VL_CLL655_CDR3-BCL1_FR4_Fwd (GTCGACCTGCAGACAGAGTTACCCGGGGCCCAACCAGCGATGGCCCAGGTTCAGCTGCAGCAGTC) and 3’ primer containing the 3’ end of the pDISPLAY cloning site, pDISPLAY_BGH_Rev (TTTTATTAGGAAAGGACAGTGGGA). Hybrid scFv mutant C was assembled by overlap-extension based on the CLL donor VL CDR3 sequence homology between the two PCR products.

Hybrid scFV mutant D: CLL donor scFv 5’ fragment was amplified using a 5’ primer containing a XmaI site, CLL655_VH_FR1_XmaI_Fwd (GTCCTCGCAACTGCCCCATCCCGGGGCCCAACCAGCGATGGCCCAGGTGCAGCTGGTGCAGTCTGG) and 3’ primer containing the BCL1 VL CDR3 sequence as overhang, VL_CLL655_FR3-BCL1_CDR3_Rev (AATAAAATGGTTGATGTACCATAGAGCACAGTAATATGTTGCAATATCTTCAGG). The CLL donor 3’ fragment was amplified using a 5’ primer containing the BCL1 donor VL CDR3 sequence as overhang, VL_BCL1_CDR3-CLL655_FR4_Fwd (GCTCTATGGTACATCAACCATTTTATTTTCGGCCCTGGGACC) and 3’ primer containing the 3’ end of the pDISPLAY cloning site, pDISPLAY_BGH_Rev (TTTTATTAGGAAAGGACAGTGGGA). Hybrid scFv mutant D was assembled by overlap-extension based on the BCL1 VL CDR3 sequence homology between the two PCR products.

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CHAPTER 4

Discussion and Future Directions

For the past four decades, monoclonal antibodies have been the dominant technological platform for producing research, diagnostic, and therapeutic tools (1). Given their remarkable parallels to gnathostome Ig-based antibodies, the discovery of LRR-based VLRB antibodies in jawless vertebrates prompted the question of whether they could also be adapted for biomedical applications. Utilizing a method to produce and screen recombinant, monoclonal VLRB antibodies developed in our lab (2), the work described in this dissertation sought to discover potentially novel tumor-associated antigens by generating and characterizing tumor-specific VLRB antibodies and their targets, and then evaluating their utility as diagnostic and tumor-targeting reagents.

Tumor antigen discovery via VLRB antibodies

At the time this work was initiated, it was observed that upon immunization with human blood group O erythrocytes or *B. anthracis* exosporium, the VLRB antibody response was mainly directed against the H-trisaccharide antigen or the BclA glycoprotein, respectively (3,4). However, we lacked detailed knowledge on the breadth of the lamprey VLRB antibody response in response to immunization with whole mammalian cells. The evolutionary distance between lamprey and mammals suggested that VLRB antibodies have the potential to recognize novel antigens that mammalian antibodies could not recognize due to structural and self tolerance restrictions. The

flipside of this hypothesis is that a ubiquitous mammalian cell surface antigen may be immunodominant, skewing the VLRB antibody response and obscuring the identification of other antigen specificities.

By characterizing the plasma VLRB reactivity of lampreys immunized with CLL cells from a patient (Chapter 2) we observed a hierarchy of VLRB reactivity led by B cells, followed by monocytes, T cells, and then NK cells (Fig 1). This suggested that the CLL immune plasma contained an array of VLRB clones that recognize different antigens, which we were able to verify by characterizing the recombinant monoclonal VLRB antibodies derived from the VLRB cDNA library of CLL immunized lamprey lymphocytes. The monoclonal VLR39 antibody proved to recognize the BCR idiotope of the patient CLL clone (Chapter 2), whereas another clone, VLR42, was found to be pan-reactive with B cells (Table 1). In a parallel study, mouse BCL1 leukemia cells were used for lamprey immunization, and a BCR idiotope-specific VLRB clone was found (Chapter 3) among 16 VLRB antibodies reactive with different lymphocyte populations. These results were in line with the different patterns of reactivity to peripheral blood mononuclear cell (PBMC) subpopulations exhibited by the 12 monoclonal VLRB antibodies derived from a human CD4+ T cell immunized lamprey (5). Our collective results thus suggest that lamprey immunization with whole mammalian cells elicits a wider spectrum of VLRB antibodies of different specificities, in comparison with the more focused VLRB antibody response generated by immunization with human blood group O erythrocytes and *B. anthracis* exosporium anthrax exosporium (3,4).

The method described in this dissertation for producing recombinant VLRB antibodies in HEK-293T cells suffers from being labor intensive and low throughput,

especially compared to yeast surface display (6,7). Individual VLRB cDNA clones are transfected into HEK-293T cells separately, and the cell culture supernatant from each transfectant is screened for antigen-specificity by ELISA or flow cytometric analysis. Each VLRB clone must go through the entire process before the antigen-specificity can be verified, and the efficiency of the process is lowered even further because less than half of the VLRB transfectants are able to secrete the VLRB protein into the supernatant. In contrast, the yeast surface display method allows the whole VLRB cDNA library to be transfected in bulk, and the entire VLRB-expressing yeast population can be enriched for the desired binding-specificities by flow cytometry and cell sorting, greatly improving the efficiency of isolating antigen-binding VLRB clones. Apart from differences in workflow, mammalian and yeast cells notably diverge in their post-translational glycosylation pathway, which may affect the proper folding, biological activity, targeting, and stability of the expressed protein (8,9).

Whether the differences between the two eukaryotic expression systems have practical consequences for finding novel tumor-specific VLRB antibodies remains an open question. Four different VLRB libraries expressed in HEK-293T cells are available for analysis. In the CLL-immune library, 36 VLRB clones were transfected, of which 14 expressed secretory VLRB protein, and 2 monoclonal VLRB antibodies with CLL reactivity were characterized (Chapter 2; Chapter 4 Table 1). In the mouse BCL1-immune library, 16 monoclonal VLRB antibodies with BCL1 reactivity were identified from screening 230 transfectants. The human CD4+ T cell-immune library yielded 11 T-cell reactive monoclonal VLRB antibodies out of 151 transfectants (5) and the *B. anthracis* exosporium-immune library yielded 14 BclA-CTD reactive monoclonal VLRB

antibodies out of 212 transfectants (2). Despite the numerical disadvantages in the number of clones that can be screened from the HEK-293T expression system, the hit rate for finding antigen-binding clones was higher than expected.

In searching for what may be a rare VLRB clone that can recognize a novel tumor epitope, it will be important to understand the factors that influence the VLRB antibody repertoire available for screening. To this end, a single source of VLRB cDNA should be expressed in mammalian and yeast cells, and their respective VLRB antibody repertoires should be examined for differences. It may be possible to improve the throughput of the HEK-293T system by expressing VLRB as membrane-bound surface molecules (mammalian cell display) (10–12) and then enriching and screening for antigen-binders in the same manner as the yeast surface display system.

The screening strategy employed in the present study ended up selecting for anti-idiotypic specificities, which by definition are tumor-specific antigens on B cell malignancies. With the knowledge that lamprey readily make anti-idiotypic VLRB antibodies, the addition of an enrichment step where the idiotypic Ig binders are subtracted should allow access to other epitopes that may be obscured by the abundance of surface Ig on B cells.

Clinical applications of anti-Id VLRB antibodies

The exquisite specificity of VLR39 for a CLL patient's BCR idiotope on the VH CDR3 was utilized to successfully detect leukemia recurrence prior to any overt clinical signs. Moreover, the detection limit of VLR39 was comparable to the current gold standard testing method of quantitative real-time PCR with V gene allele-specific primers

(Chapter 2). Another monoclonal VLRB antibody, VLR-C8, also recognized a BCR idiotope comprised of the VH/VL CDR3.

In contrast to previous reports that VLRB antibodies isolated directly from the library tend to be of low affinity without *in vitro* affinity maturation (2,5,6,13), both VLR39 and VLR-C8 could be expressed as monomeric VLRB proteins which were capable of binding to their cognate BCR idiotope without additional mutagenesis to improve affinity (Chapter 3 Fig 5; Chapter 4 Fig 2). It is tempting to speculate that the molecular flexibility of the Ig CDR loops and the extended LRRCT loop of the VLR cause both structures to adopt conformations with exceptional shape complementarity, though contributions from the rigid, concave LRRV surface should also be taken into account.

The VTVKYYDFWGY amino acid sequence of the CLL patient VH CDR3 by itself is sufficient to confer VLR39 binding, which suggests that VLR39 likely recognizes a linear epitope (Chapter 2, Fig 2). On the other hand, the interaction between VLR-C8 and the VH/VL CDR3 of the mouse BCL1 Ig is not as clear cut (Chapter 3, Fig 4). X-ray crystallography of the VLR39 in complex with CLL patient Ig may shed light on how a VLRB protein interacts with a long, flexible loop structure unlike any of the VLRB-Ag molecular interfaces described previously (13–16).

The apparent immunogenicity of Ig idiotopes in lampreys suggests that the method described in this dissertation may offer an alternative option for generating anti-idiotypic reagents that possess specificity and affinity on a par or better than most anti-idiotypic Ig antibodies (17,18). Readily available anti-Id antibodies may facilitate the

study of clinical cancer dormancy in B cell malignancies (19) by allowing for the capture and study of residual leukemic clones in patients during clinical remission.

It may be impractical to raise highly specific anti-Id antibodies for each individual CLL patient for diagnostic and monitoring purposes. However, up to a third of CLL patients share very similar VH CDR3 sequences and *IGHV* genes or gene families (20); these remarkably similar antigen binding sites are termed stereotyped BCRs (21). Stereotyped BCRs occur much more frequently among patients with unmutated *IGHV*s, who characteristically have the worst clinical outcomes, and certain stereotypes correlate with distinct clinical course and outcomes (22). A panel of antibodies reactive with stereotyped BCRs would thus be useful for targeted diagnostic and therapeutic uses in a significant number of CLL patients, either as soluble effectors or as antigen receptors with exquisite specificity for tumor cells on chimeric antigen receptor T cells (23,24). Because the level of circulating tumor-specific Ig in CLL is lower than in many B cell malignancies, target inhibition by soluble Id is much less likely to reduce the efficiency of such a therapeutic approach. Furthermore, the same antibody that would be used for treatment could also be used to predetermine the levels of circulating CLL Ig in the patient's serum. If this level was deemed too high, an initial "de-bulking" regimen could be used to lower the number of leukemic cells and reduce the serum levels of CLL Ig. Finally, the same antibodies could potentially be used for remission induction therapy or as a consolidation treatment to eliminate residual leukemia cells.

It has been difficult to raise anti-stereotype antibodies by immunization of mice, and this difficulty is thought to be due to self-tolerance constraints owing to the sequence similarity between murine and human *IG* genes. The lack of *IG* genes in lamprey may

circumvent the self-tolerance constraints and permit recognition of shared mammalian epitopes. This possibility is supported by the ability of VLR39 to discriminate effectively between CLL donor and other CLL cells with BCRs of very similar amino acid sequences (Chapter 2). The ability of lampreys to produce VLRB antibodies recognizing stereotyped BCRs could therefore provide an attractive means for developing useful reagents for monitoring groups of CLL patients. Lamprey LRR-based anti-Id antibodies thus offer a complementary approach to the use of classical Ig-based anti-Id antibodies in the monitoring and management of patients with CLL.

Tumor-targeting VLRB antibodies

The limitation of the IgG format has driven the search for innovative engineered proteins with new capabilities, especially for *in vivo* applications. Several desired qualities come up repeatedly: small size for improved tumor penetration, simplified and stable structure amenable to cheap manufacture by prokaryotic expression or chemical synthesis, and flexibility to modify valency, function, and pharmacokinetics (25,26). VLRB antibodies fit some of these criteria, and miss on others. The monomeric form of VLRB is relatively small at 15~25 kDa comparable with nanobodies, although not as small as peptides and small molecule drugs. VLRB antibodies are stable under harsh conditions (4) and have been expressed in bacteria, although as inclusion bodies that require *in vitro* folding due to crucial disulfide bonds. The single polypeptide VLRB is amenable to modification by recombinant molecular techniques or biochemistry.

The generation of the high affinity anti-BCL1 idiotope VLR antibody described in Chapter 3 allows for the characterization of VLRB as an *in vivo* tumor targeting reagent

in the context of the syngeneic mouse BCL1 leukemia model (27–29). In comparison to tumor models that involve xenograft in immunocompromised mice, the intact immune system in the BCL1 model will also allow for the evaluation of dimeric VLR-Fc fusion proteins to elicit CDC/ADCC, and immunogenicity.

Preliminary experiments indicate that intravenously injected monomeric VLRB is metabolized and excreted rapidly; 24 hours after injection, VLRB monomers were only detected in the gastrointestinal tract (my unreported observations), which suggests that clearance is mediated by biliary exrection. This was somewhat unexpected, as the small size of the VLRB protein should allow for clearance through renal filtration (30). These results are in concordance with the safety record of non-Ig protein scaffolds in clinical trials (31–36), which can be attributed to rapid tissue clearance owing to their small size.

The use of non-human biologics elicit concerns about immunogenicity, which can have adverse effects on the patient in the form of infusion reactions, anaphylaxis, and immune-complex mediated disease (37) as well as loss or change in drug efficacy due to altered pharmacodynamics and pharmacokinetics via anti-drug antibodies (ADA). The very fact that mouse anti-VLRB monoclonal antibodies have been made (3,38) show that VLRB is immunogenic under the right conditions. However, even an antibody with fully human sequence cannot eliminate immunogenicity, as documented by the 12% incidence of ADA in patients administered fully human anti-TNFα adalimumab (Humira) without additional immunosuppression (39).

As with all the other engineered proteins, the advantages and disadvantages of any given protein will be weighed against its intended use. Given the unique structural features honed over 500 million years of evolution, the VLRB antibody has a very high

likelihood of finding and binding to antigens impossible or difficult for other protein scaffolds, and may find clinical application “…in the hands of the physician a victorious weapon against illness and deaths" (40).

Figures and Legends

Figure 1

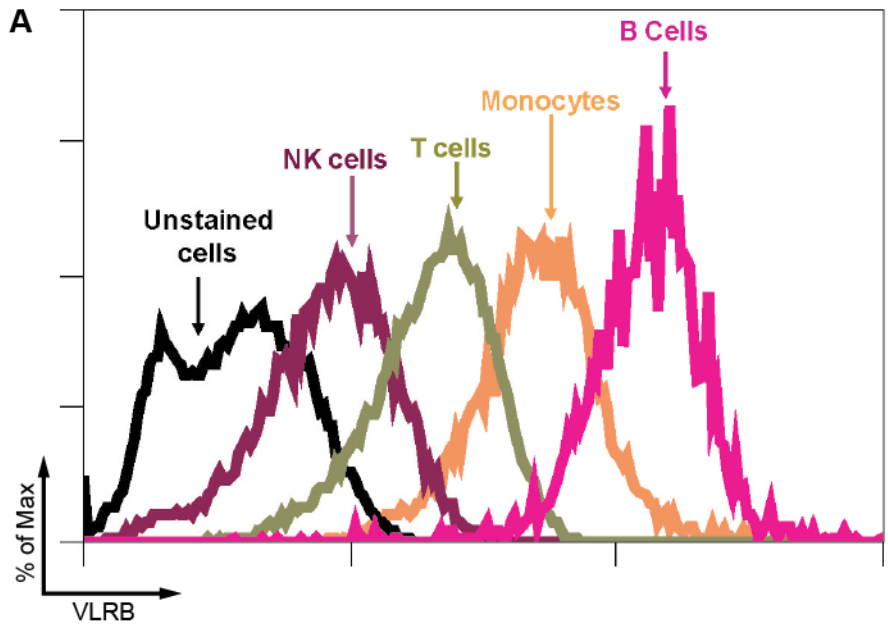

Figure 1: Flow cytometric analysis of plasma from CLL immunized lamprey. PBMC from healthy adults were sequentially incubated with CLL-immune lamprey plasma, followed by 4C4 anti-VLRB mAb, and then RPE-conjugated goat anti-mouse IgG polyclonal antibodies, before blocking with 5% mouse serum and staining for T cells (CD3), B cells (CD19), monocytes (CD14) and NK cells (NKp46). Cells were washed in PBS between incubation steps and propidium iodide added before flow cytometric analysis of the cells.

Table 1. Screening results of monoclonal VLRB antibodies from CLL immunized lampreys.

	VLR39	VLR42
CLL ID		
Donor	+	+
Donor (Remission; CD19+)	-	+
33	-	+
37	-	+/-
74	-	+
90	-	++
B cell lines (EBV transformed)		
WT4125	-	++
WT4346	-	++
M4017	-	++
M4107	-	++
Leukemias/Lymphomas	-	
Nalm-16 (pro-B)	-	-
697 (pre-B ALL)	-	-
Daudi (Burkitt's)	-	+/-
Ramos (Burkitt's)	-	-
SU-DHL-6 (Diffuse large B cell)	-	+
NCI-H929 (Multiple myeloma)	-	-

Table 1: Screening results of monoclonal VLRB antibodies from CLL immunized lampreys. VLR39 and VLR42 reactivity against CLL, EBV transformed B cell lines, and various B cell leukemia and lymphoma lines was compared with that of control VLR4 having *Bacillus anthracis* BclA-specificity by flow cytometric analysis. The plus and negative signs indicate difference in staining intensity compared to negative control as follows: (+/-) less than 0.5 log shift, (+) >0.5 and <1.5 log shift, (++) > 2 log shift.

Figure 2

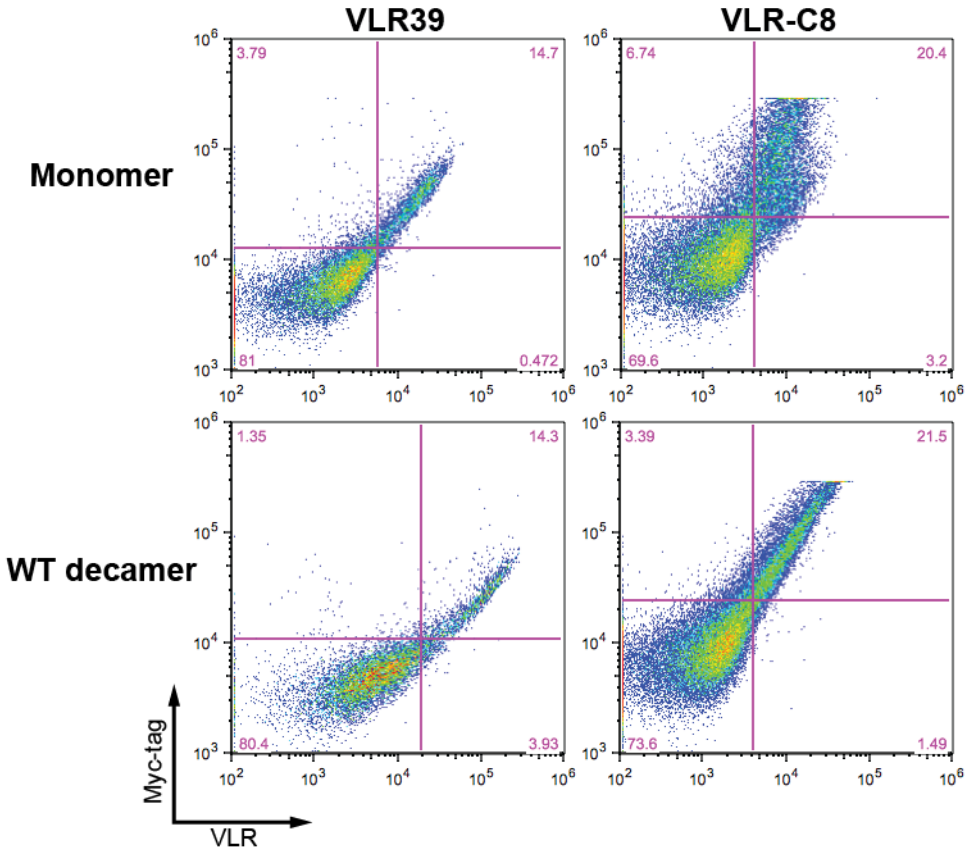

Figure 2: Flow cytometric analysis of anti-Id VLRB antibodies in monomeric form. VLR39 and VLR-C8 monomer (top row) binding to CLL scFv and BCL1 scFv respectively, was compared with that of VLR39 and VLR-C8 wildtype decamer (bottom row). Myc-tag was co-stained to measure scFv surface expression.

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