Curr Opin Biotechnol. 2018 August ; 52: 74–79. doi:10.1016/j.copbio.2018.02.016.

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adnectins, anticalins, and cysteine knot proteins, among others [1–6]. This review will focus on a relatively new antibody alternative based on a class of natural antigen binders found in jawless vertebrates known as variable lymphocyte receptors (VLRs) and describe recent engineering and therapeutic applications.

VLR biology and structure

VLRs are part of the adaptive immune system of jawless vertebrates. Early evidence indicated that lamprey and hagfish had an adaptive immune system; and while they had cells that resemble mammalian lymphocytes, no immunoglobulin, T cell receptor, or major histocompatibility complex genes could be identified [7–9]. Instead, Pancer and colleagues identified VLRs as the antigen receptors in activated lamprey lymphocytes [10]. In contrast to the mammalian immunoglobulin fold, VLRs are leucine rich repeat (LRR) proteins that generate diversity by a combination of differential repeat number and sequence variability [10,11] (Figure 1). In a bit more detail, three different VLR genes, *VLRA*, *VLRB* and *VLRC* have been identified in jawless vertebrates, with *VLRA* and *VLRC* being expressed in lymphocytes that resemble T cells and *VLRB* expressed in lymphocytes resembling B cells [12,13]. Since *VLRB* is expressed in a soluble format by B cell-like lymphocytes which respond to an adaptive challenge, *VLRB* is the most widely used form for VLR applications. VLR diversity is generated by gene conversion-type mechanisms [10,11] which yield VLRs comprising an N-terminal cap (LRRNT), the first LRR (LRR1), up to seven variable LRR (LRRVs), an end LRR (LRRVe), a connecting peptide (CP), a C-terminal cap (LRRCT), an invariant stalk, a glycosylphosphatidylinositol (GPI) anchor, and a cysteine rich hydrophobic region that can drive VLR multimerization [14,15] (Figure 1A). These modules combine to form a crescent-shaped solenoid structure with a concave surface consisting of parallel β -sheets which forms the antigen binding site [16,17] (Figure 1). Within the antigen binding site, there exist highly variable residues with each interfacial LRRV β -sheet having 6 residues of the general form $X^1LX^3LX^5X^6$ [18,19]. The leucine residues (L) form the hydrophobic core and the variable amino acids (X) generate the unique antigen binding interface (Figure 1B). Soluble VLRs also multimerize by forming disulfide bonds via a cysteine-rich hydrophobic region [14], but VLRs can also be used in monomeric forms for biotechnology purposes by expression without the hydrophobic region. Thus, VLRs represent an alternative antigen receptor, with many parallels to mammalian antibodies, that can combine with antibody engineering platforms to address both traditional and unique challenges.

Design and selection of VLR binders

For selection of VLRs that bind the antigen of interest, nonimmune, immune or designed libraries can be employed. Since VLRs are natural antigen binders, they have evolved to have a potential diversity of 10^{14} [15]; and thus, the nonimmune VLR repertoire can be used to identify specific antigen binders [19,20]. Another option is lamprey or hagfish immunization with a desired antigen to produce a diverse immune library. As a result of substantial evolutionary distance from mammals, VLRs may be advantageous for recognition of conserved mammalian epitopes or glycans that may be troublesome for Ig-based systems because of tolerance mechanisms, particularly those relying on immunization

techniques. Particulates and cells work well for lamprey or hagfish immunizations; however, soluble proteins often require an adjuvant or be displayed on a cell surface to elicit a robust immune response [12,21]. Using these strategies, immunized libraries have also been used successfully to identify antigen binders [12,14,21,22]. Typically, the VLRB cDNA which encodes for the soluble form of VLRs is recovered from the lymphocytes and cloned into a surface-display platform for screening. To date, most studies have employed the monomeric VLR form spanning from LRRNT to the LRRCT (Figure 1). After VLR identification, the multimerizing tail can be reintroduced to increase the avidity for lower affinity clones.

Consensus VLR scaffolds represent another emerging option for deploying VLR technology. Scaffolds known as dVLR and repebody represent two examples that are based on a VLR consensus sequence. First, a designed VLR (dVLR) was developed by choosing the most frequently encountered amino acid at each position of aligned lamprey VLRB sequences, and the dVLR was designed with just 1 LRRV module since the average usage in lamprey VLRBs is 1.3 LRRVs [23,24]. This scaffold was produced solubly in bacteria and showed excellent stability over a broad range of pH range temperature. A combinatorial library was produced by randomizing 11 highly variable residues in the contact interface of LRR1, LRRV and LRRVe and screened to identify VLRs capable of binding lysozyme and S100A7 protein [24]. The repebody is another consensus VLR-based scaffold comprising 5 consensus LRRV modules, based on the number of LRRVs in known antigen binding VLRs, and with an internalin B cap replacing the LRRNT module for better expression in bacteria [25]. After randomizing three variable residues in both LRRV1 and LRRV2, libraries were screened to identify repebodies that bind to IL-6 [25], among other targets, and as discussed in more detail below. Finally, recent computational design strategies employing Rosetta techniques have identified VLRs similar to those that are naturally occurring in addition to the designed VLRs [26]. Importantly, due to the modular nature of VLRs, the number of LRRVs can be altered, as exemplified by the two consensus VLR scaffolds, to accommodate antigens of different chemistry and sizes. Once a VLR library is created, it can be screened using a surface display platform including phage [24,25,27], and yeast display [20,21,28]. Soluble VLRs have been expressed in bacteria, mammalian cells, and even plants for further evaluation. Then, specific VLRs can undergo random or targeted mutagenesis of the concave surface to improve affinity. For example, the IL-6 repebody was subjected to affinity maturation by mutagenesis of the LRRV variable positions to yield a 63 pM binder [29].

Antigens successfully targeted by VLRs

Once VLR libraries are in hand, they can be screened against a wide variety of antigens. Like antibodies, VLRs can bind protein antigens with high affinity and specificity. Antigenic proteins have been sourced from mammalian cells (hen egg lysozyme [18] and CD38 on plasma cells [30]), bacteria (spore surface protein BclA [14] and plant pathogen protein HopM1 [28]), and viruses (avian influenza virus hemagglutinin [22]). An example of the high specificity that can be achieved with VLRs can be found in the identification of a panel of VLRs that recognized BclA on *Bacillus anthracis* spores but not on *Bacillus cereus* despite 89.5% sequence identity [14]. VLRs have also been proven adept at binding glycans such as Thomsen-Friedenreich antigen and H-trisaccharide, with high specificity and affinity [12,20]. Since jawless vertebrates elicit a strong VLR response to particulates, they can be

used to identify cell-surface biomarkers. As an example, it has been difficult to identify antibodies targeting specific surface markers on plasma cells (PCs), likely a result of mammalian immune tolerance. Using the VLR repertoire from a bone marrow aspirate-immunized lamprey and clonal evaluation, a VLR targeting CD38 was identified that exclusively bound PCs [30].

Antigen recognition by VLRs

Even at this somewhat early stage in VLR applications, VLRs have been identified against a reasonable array of antigens including glycans. The broad applicability of VLR approaches could be in part due to the mode of VLR binding, which has recently been partially elucidated using the crystal structures of VLRs in complex with antigens [14,16,18,19,31,32]. Based on the available structural analyses, it appears that large antigens like proteins contact all three “ridges” formed by the variable residues in the concave interface [18,19] (Figure 1B). Interestingly, smaller antigens like glycans contact the bottom two ridges of the interface [19]. In addition to the LRRVs, a variable-sized insert in the LRRCT that can contribute to binding. This variable insert is structurally diverse forming β -hairpins or loops and in some cases, no insert is present [16]. The LRRCT insert has been shown to sandwich glycans, H-trisaccharide and Thomsen-Friedenreich antigen, against the concave binding interface [18,19] (Figure 1B). The LRRCT insert has also been shown to insert in the cleft of HEL [18], bind a flat surface of HEL [32], or lay in a shallow groove of BclA [31]. Taken together, the LRRCT exhibits an impressive contribution in terms of binding versatility and may help aid in the recognition of diverse antigen types. The structural knowledge of the binding interactions should further enable rational design of consensus VLR libraries and/or affinity maturation. However, much like antibodies, the variable contact residues are not the only factors driving binding affinity. For instance mutation of non-contact residues that promoted better shape and electrostatic complementarity caused a 13-fold affinity increase for an anti-HEL VLR [18].

Engineering of VLRs for downstream applications

Therapeutic applications

Given their targeting capability, VLRs have been engineered for different therapeutic applications (summarized in Table 1). First, in a direct binding format, an evolved high affinity IL-6-binding repebody could inhibit IL-6 induced signaling in non-small cell lung cancer cells and suppress their growth in xenograft models [29]. As additional repebody examples, a C5a repebody could suppress proinflammatory response [27], and an anti-VEGF repebody could block neovascularization and vascular leakage in a mouse model of age-related macular degeneration [33]. Another approach is to employ a VLR as the recognition domain of a chimeric antigen receptor (CAR), and Moot and colleagues successfully created VLR-CARs that were expressed on T cells and could mediate cytotoxicity of the target cell [34]. VLRs have also been used in drug delivery applications. For example, an anti-tumor reagent was chemoenzymatically attached to the C-terminal end of the EGFR-specific repebody, and the drug conjugate elicited tumor regression in xenografted mice [35]. The same EGFR-specific repebody was also fused to a tumor

apoptosis protein, apoptin, that assembled into stable nanoparticles providing anti-tumor activity in mouse xenografts [36]. Therapeutic application of VLRs is very promising; however, since VLRs are derived from jawless vertebrates, they could be immunogenic. Along these lines, preliminary evaluation of the anti-IL-6 rebody indicated negligible immunogenicity [29], although this issue warrants further attention by the field.

Diagnostic applications

As discussed earlier, a VLR was identified that exclusively bound PCs via CD38 interactions. This VLR was capable of identifying healthy PCs and many multiple myelomas [30], suggesting that this VLR could be used as a diagnostic reagent. Another diagnostic demonstration employed the anti-Thomsen-Friedenreich antigen VLR to determine that non-small cell lung cancer patients that stained with the VLR had a worse survival prognosis [20]. Similarly, an anti-idiotypic VLR could be used to monitor recurrence of chronic lymphocytic leukemia [37]. VLRs could also be valuable imaging reagents as demonstrated for EGFR-specific rebody employed for *in vivo* imaging EGFR-expressing tumors [38,39] and mCherry-specific rebody employed for tracking protein-protein interaction in live cells [40]. VLRs could also be used to study plant pathology and target disease forming pathogens. A VLR against a plant pathogen, HopM1, was able to be expressed and interact *in planta* [28].

Other applications

Non-binding VLR components have also been used in several ways. VLR residues have been used to stabilize other LRR proteins. For example, a murine neuronal adhesion molecule and LRR protein, LRRTM2, failed to form diffraction quality proteins. When LRRTM2 was stabilized using the lamprey VLR consensus sequence to mutate 33% of its amino acids, mainly located on the convex, non-binding surface, the crystal structure could be resolved [41]. Another approach to increase the stability of LRR proteins is to use the LRR hybrid technique [42]. LRRNT and LRRCT caps of hagfish VLR were grafted onto the ends of LRR toll-like receptors to solubilize and stabilize them for crystallization studies [42,43]. Another non-binding application comes from the multimerization capability of the hydrophobic region. To this end, the C-terminal multimerizing end of VLRB was fused to cancer relevant vaccine targets, creating a multimeric vaccine with minimal exposed VLR sequence. These properties resulted in a more effective vaccine with higher Ab titer against the vaccine target and lower Ab titer against the VLR component compared with other carrier strategies [44].

Conclusion

Despite their relatively recent discovery, VLRs have already been demonstrated to be a powerful antibody alternative. As described above, the crescent-shaped VLR solenoid has a highly variable concave binding surface that combines with the LRRCT insert to provide a versatile binding interface that results in highly specific binders to proteins and glycans. Like other alternative scaffolds, VLRs possess high thermal and pH stabilities and can be readily multimerized and engineered [14,23]. However, given that VLRs are bonafide antigen receptors, they are additionally compatible with immunization methods that help

generate binding reagents with high specificity and affinity. Compared with mammalian antibodies, the high affinities and specificities of VLRs for glycans offer a potentially intriguing advantage, and VLRs should be explored further as novel targeting reagents that could be used to discover and/or monitor glycosylation signatures characteristic of cell types or disease. Another potential niche for VLRs versus mammalian antibodies leverages the evolutionary distance of jawless vertebrates from mammals. In particular, VLRs could be used to target highly conserved mammalian proteins, a process that is often difficult using Ig-based technologies. Finally, the multimeric structure may prove advantageous for the rapid tuning of valency and provide higher avidity for applications that require highly sensitive targeting.

Acknowledgments

This work was supported by National Institutes of Health grants NS091851 and NS099158 to E.V.S.. E.A.W. is supported by the National Human Genomes Research Institute training grant to the Genomic Sciences Training Program 5T32HG002760. Virus and spore images in the visual abstract were adapted from Dan Higgins and Janice Haney Carr of the Centers for Disease Control and Prevention.

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This study demonstrates a unique non-binding application of VLRs and the utility of the multimerization domain.

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Highlights

- VLRs bind proteins and glycans with high specificity and affinity.
- Lamprey or hagfish immunization produces diverse VLR repertoires.
- Modular engineering of VLR scaffolds is possible.
- VLRs have been engineered for a variety of therapeutic applications.

Figure 1.

Overview of VLR structure. (A) Ribbon structure of a monomeric VLR in complex with an H-trisaccharide (gray) (PDB code 326J [16]). Below the ribbon structure is a modular rendition of a full-length VLR consisting an N-terminal cap (LRRNT), the first leucine-rich repeat (LRR) module (LRR1), three variable LRR (LRRVs), an end variable LRR (LRRVe), a connecting peptide (CP), a C-terminal cap (LRRCT), an invariant stalk, a glycosylphosphatidylinositol (GPI) anchor, and a hydrophobic region. (B) The VLR structure is rotated 90° with highly variable residues of the 6-residue motif ($X^1LX^3LX^5X^6$) in purple and the variable sized insert of the LRRCT in dark blue. X represents highly variable amino acids and L, leucine.

Table 1**Applications of VLRs in Biomedical Research**

	Application	VLR function	Result	References
Therapeutic targeting	Inhibit signaling pathway	IL6 binder	Inhibits IL-6 induced signaling pathway in non-small cell lung cancer cells	[29]
	Inhibit signaling pathway	C5a binder	Inhibits C5a pathway to suppress proinflammatory response	[27]
	Inhibit signaling pathway	VEGF binder	Blocks the VEGF signaling pathway for age-related macular degeneration	[33]
Drug delivery	CAR-VLR	B-cells and CD5 binder	Expresses on effector cells, redirect to target cells, and activates cytotoxicity	[34]
	VLR-drug conjugate	EGFR binder	Chemoenzymatically attaches drug for tumor suppression	[35]
	Nanoparticle	EGFR binder	Self-assembled nanoparticles target tumors	[36]
Diagnostics/Imaging	Biomarker discovery	CD38 binder	Novel epitope that exclusively binds PCs	[30]
	Diagnostics	TF- α binder	Stains tumor tissue with worse survival rate	[20]
	Diagnostics	Anti-idiotypic binder	Used to monitor lymphocytic leukemia	[37]
	Molecular imaging	EGFR binder	In vivo tumor imaging	[38,39]
	Molecular imaging	mCherry binder	Tracks protein-protein interaction in real time	[40]
	Plant research	HopM1 binder	Expresses and binds in some plant environments	[28]
Other	Stabilization	Consensus sequence	Able to crystalize LRRTM2 by altering convex surface based on VLR sequence	[41]
	Stabilization	Stable caps	Resolves crystal structure of LRR proteins using caps comprised of VLR sequence	[42,43]
	Vaccine carrier	Multimerizing tail	Creates multimer with hidden VLR sequence resulting in high vaccine Ab titers	[44]