Structure reveals function of the dual variable domain immunoglobulin (DVD-Ig™) molecule

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Abbreviations: DVD-Ig™, Dual-variable Domain immunoglobulin™ molecule; mAb, monoclonal antibody; IgG, Immunoglobulin G; Fab, Antigen binding Fragment of an immunoglobulin; DFab, DVD-Ig™ Fab fragment; IL12 and IL18, Interleukins 12 and 18; VD, VD1, and VD2, Variable domain, outer and inner variable domain; C_H, Constant heavy and constant light chain domains; V_h and V_l, Variable heavy and constant light chain domains; CDRs, Complementarity determining regions; Sc, Surface complementarity statistic; rmsd, Root-mean-square deviation

Several bispecific antibody-based formats have been developed over the past 25 years in an effort to produce a new generation of immunotherapeutics that target two or more disease mechanisms simultaneously. One such format, the dual-variable domain immunoglobulin (DVD-Ig™), combines the target binding domains of two monoclonal antibodies via flexible naturally occurring linkers, which yields a tetravalent IgG-like molecule. We report the structure of an interleukin (IL)12-IL18 DVD-Ig™ Fab (DFab) fragment with IL18 bound to the inner variable domain (VD) that reveals the remarkable flexibility of the DVD-Ig™ molecule and how the DVD-Ig™ format can function to bind four antigens simultaneously. An understanding of how the inner variable domain retains function is of critical importance for designing DVD-Ig™ molecules, and for better understanding of the flexibility of immunoglobulin variable domains and linkers, which may aid in the design of improved bi- and multi-specific biologics in general.

The bispecific tetravalent immunoglobulin known as the dual variable domain immunoglobulin or DVD-Ig™ molecule was first described by Wu et al. in 2007. Like a conventional IgG, the DVD-Ig™ molecule is composed of two heavy chains and two light chains. Unlike IgG, however, both heavy and light chains of a DVD-Ig™ molecule contain an additional variable domain (VD) connected via a linker sequence at the N-termini of the VH and VL of an existing monoclonal antibody (mAb). Thus, when the heavy and the light chains combine, the resulting DVD-Ig™ molecule contains four antigen recognition sites (Fig. 1). The outermost or N-terminal variable domain is termed VD1 and the innermost variable domain is termed VD2; the VD2 is proximal to the C-terminal CH1 or CL. We and others have previously reported that DVD-Ig™ molecules can be manufactured and purified to homogeneity in large quantities, have pharmacological properties similar to those of a conventional IgG, and show in vivo efficacy in multiple mouse models.1,2

Theoretically, the structurally novel DVD-Ig™ format design can impose certain structural and functional constraints on VD2, the inner variable domain. In a conventional mAb, the complementarity-determining regions (CDRs) of a VD are surface exposed with no N-terminal constraints, no limitations as to the size and location (soluble or cell surface) of the target antigen, and no constraints as to conformational changes that might occur upon target binding (VD stabilization). In the DVD-Ig™ molecule, the juxtaposition of VD1 to VD2 via linkers could potentially occlude VD2 CDRs, limit VD2 rotational flexibility, impose limits on target size and location, or impose constraints on VD2 conformational changes (stabilization) upon target binding. We and others, however, have observed that affinity at the inner antigen binding site (VD2) may be somewhat dependent on the VD1/VD2 pair combination (amino acid sequences), the VD1/VD2 orientation, and linker selection.2,3 The choice of linker length between the VD1 and VD2 [e.g., either both short linkers (S-S), both long linkers (L-L), or one short and one long linker (S-L or L-S) as shown in Table 1], can affect the affinity of the inner VD2 domain.4 In addition, the antigen affinity at the outer antigen binding domain (VD1) is often nearly equal to the parent antibody in the DVD-Ig™ format. Both VD1 and VD2 of a DVD-Ig™ molecule can successfully target soluble and cell surface antigens and can bind antigens simultaneously and with full occupancy.5 Here, we report the structure of the interleukin (IL)12-IL18 DVD-Ig™ Fab with IL18 bound at VD2. The structure provides the basis for understanding how the DVD-Ig™ molecule binds two different antigens simultaneously and lays

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the foundation for hypothesis-driven design of new DVD-Ig™ molecules with adjustable binding properties.

An initial view of the DVD-Ig™ DFab structure immediately reveals how the DVD-Ig™ molecule functions to bind two different antigens on each DFab simultaneously (Fig. 1B and C). With IL18 bound to VD2 (the inner variable domain), the outer VD1 rests entirely on top of the heavy chain of the inner variable domain. This orientation of the outer variable domain positions the CDRs of VD1 for binding the second antigen approximately 85° from the inner antigen CDRs (Fig. 2A), leaving ample room for binding the outer antigen (IL12). The structure helps explain how the binding affinity for both antigens in this DVD-Ig™ molecule remains essentially unchanged from the parent antibodies [K_D (pM) mAbs: IL12 = 120, IL18 = 140; IL12-IL18 DVD-Ig™: IL12 = 130, IL18 = 160].

Table 1. DVD-Ig™ linkers

| Linker Nomenclature | Linker Sequence and Combination |
|---------------------|---------------------------------|
| Short-Short (SS)*   | ASTKGP (6a.a.) TVALP (5a.a.)   |
| Long – Long (LL)    | ASTKGSVPLAP (13a.a) TVALSVFIPF (12a.a.) |
| Long – Short (LS)   | ASTKGSVPLAP (13a.a) TVALP       |
| Short – Long (SL)   | ASTKGP (6a.a) TVALSVFIPF (12a.a.) |

*Linkers used for IL12-IL18 DVD-Ig™ molecule construction.

Having two linkers to the outer variable domain in the DVD-Ig™ format affords additional flexibility when engineering DVD-Ig™ molecules. By adjusting the length and sequence of the linkers, the position of the outer variable domain may be altered, making it possible to tune the affinity of the inner antigen site. A cartoon schematic illustrating how varying the linker length can reposition the outer VD to improve interactions with the inner VD is shown in Figure 3A. We have seen a significant loss of affinity for vascular endothelial growth factor (VEGF) when it is expressed at the inner VD2 with SS linkers (Table S2).

To investigate the effect of linker length and outer VD sequence pairing on inner VD antigen affinity, we constructed a matrix of DVD-Ig™ proteins pairing anti-VEGF VD with four different paratopes of an alternate therapeutic target of 54.7 kDa that we refer to as AgA. These DVD-Ig™ molecules were made with four different linker sets, short-short (SS), short-long (SL), long-short (LS), and long-long (LL), as described in Table S2. The VEGF binding kinetics for this series of DVD-Ig™ proteins suggest that the affinity for the inner antigen can be modulated by adjusting linker length, and that it is possible to screen for desired linker length combinations. The binding kinetics (Fig. 3B) reveal a linker length dependent reduction of VEGF affinity (K_D) for the inner VD that is primarily the result of reduced on-rate (k_on) for short linkers, relative to the reference IgG. Furthermore, the binding kinetics show that the K_D can be modulated by adjusting linker length and by using different outer VD sequences. Overall, we can always identify a DVD-Ig™ molecule retaining binding affinities to both inner and outer variable domains through a process of selecting the best inner/outer variable domain combination, optimizing the orientation of the two variable domains and adjusting the linker length and type. The ability to modulate the

Table S2

| Linker | Linker Sequence and Combination |
|--------|---------------------------------|
| Short-Short (SS) | ASTKGP (6a.a) TVALP (5a.a) |
| Long – Long (LL) | ASTKGSVPLAP (13a.a) TVALSVFIPF (12a.a) |
| Long – Short (LS) | ASTKGSVPLAP (13a.a) TVALP |
| Short – Long (SL) | ASTKGP (6a.a) TVALSVFIPF (12a.a) |

*Linkers used for IL12-IL18 DVD-Ig™ molecule construction.
affinity of one antigen with respect to the other may be an important feature for any dual specific binding protein when dosing two paratopes simultaneously. Although it is not necessary for the proper function of a DVD-Ig™ molecule, in certain applications one or both of the linkers can be particularly engineered to be enzymatically cleaved to enhance or activate antigen binding at the inner antigen site. Cleavage of one linker allows outer VD1 additional rotational freedom, and greater accessibility to the inner VD2 antigen binding site. This feature may enable tissue targeting of the DVD-Ig™ molecule with the outer variable domain while masking the paratope of the inner domain until the DVD-Ig™ molecule is “activated” at the targeted site by tissue specific proteases. We have recently shown that a DVD-Ig™ molecule that substantially lost antigen affinity at the inner VD2 specific sites. Challenges in developing therapeutic bispecifics, however, remain, e.g., molecular stability, pharmacokinetics, manufacturability of individual bispecific formats. Of the more than 30 bispecific formats described to date, there are no crystal structures reported for any other bispecific format that reveals both paratopes with an antigen bound. This fact speaks to the robust nature of the DVD-Ig™ format and the ability to produce these proteins in large quantities in a highly purified and stable form. The DVD-Ig™ format is a novel immunotherapeutic platform that exploits the benefits of the immunoglobulin fold through optimization of its various components while engineering in the necessary flexibility to bind an additional antigen. This platform can be utilized to bind a combination of both soluble or cell surface expressed antigens simultaneously. The information contained within the structure of the complex of the DFab with IL18 has proven to be particularly useful for understanding antigen binding properties of other DVD-Ig™ protein pairs and for designing future DVD-Ig™ molecules. There is obviously a need for additional structural studies with a variety of antigens at both the inner and outer variable domain positions in order to more fully comprehend the dynamic nature of the DVD-Ig™, in particular how the various states of antigen occupancy affect VD positioning and how the different antibody frameworks interact with one another and reposition upon antigen binding. It is expected that this structure and future structures will facilitate the design of a new generation of dual specific therapeutics.

Materials and Methods

Protein production, purification and crystallization. All DVD-Ig™ molecules were prepared as previously described. DFab was prepared from the DVD-Ig™ molecules by standard papain digestion and purified using high performance ion exchange and gel filtration. A three-fold excess of IL18 was used to make the complex. The complex was separated from excess IL18 by gel filtration, concentrated to 17 mg/ml, and crystallized by vapor diffusion in a 1:1 ratio against a reservoir containing 1 mL of 2 M ammonium sulfate, 0.1 M sodium acetate pH 4.6 at 4°C. Crystals were flash frozen in liquid nitrogen using reservoir solution and 25% (v/v) glycerol.

X-ray data collection, structure solution and analysis. Data were collected at 100 K using 1 Å wavelength at the Advanced Light Source 502 beamline to 2.8 Å and processed using HKL2000. Initial phases were obtained by molecular replacement using the Fab, and VD from pdb code 2XV7 and IL-18 from pdb code 2XVT using the programs Phaser and MolRep (CCP4 suite of programs). A randomly selected set of 5% of total reflections was used for Rfree calculations. Iterative cycles of refinement and model building were performed using Refmac17, AutoBuster,18 and Coot.19 A second data set was collected in a similar fashion using the Advanced Photon Source IMCA 17-ID beamline on a
single crystal which grew after 22 mos and diffracted to 2.1 Å. The data were processed using AutoProc\textsuperscript{21} and refined as above. The quality of the final structure was evaluated using programs within the CCP4 suite with 98.7\% of all residues in favored or allowed regions of the Ramachandran diagram. Final refinement statistics can be found in Table S1. All alignments were performed using SSM superpositioning in Coot;\textsuperscript{19,20} electron density maps were calculated using CCP4\textsuperscript{16} the surface areas and complementarity statistics were calculated with the programs AREAIMOL and SC\textsuperscript{5} respectively (CCP4\textsuperscript{16} program suite); and all molecular figures were generated using the program PyMol.\textsuperscript{22}

**Binding analysis.** Protocols for SPR binding studies and individual sensograms are available in the online Supplemental information.
The first data set was collected at the Advanced Light Source, Berkeley, CA. Final X-ray diffraction data were obtained at the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source. These facilities are supported by the companies of the Industrial Macromolecular Crystallography Association.

We thank Chengbin Wu and Hua Ying formerly of Abbott Bioresearch Center for making the IL12-IL18 DVD-Ig™ construct; Alexander Ivanov, George Cunha, Carrie Goodreau, David Lee, and Georgeen Gaza-Bulseco of AbbVie Bioresearch Center for their contributions to preliminary DVD-Ig™ analyses; Virginia Rath (Reciprocal Space Consulting, L.L.C., Oakland, CA) for the X-ray diffraction data collection for Figure 3.

**Table 2.** DVD-Ig™ binding kinetics for AgA and VEGF

| Symbol | mAb / DVD | VD1 | VD2 | Linker HC/LC | k<sub>a</sub> (M<sup>-1</sup> s<sup>-1</sup>) | k<sub>d</sub> (s<sup>-1</sup>) | K<sub>d</sub> (M) | k<sub>a</sub> (M<sup>-1</sup> s<sup>-1</sup>) | k<sub>d</sub> (s<sup>-1</sup>) | K<sub>d</sub> (M) | K<sub>d</sub> ratio (DVDFparental) |
|--------|-----------|-----|-----|--------------|-----------------|-----------------|-----------|-----------------|-----------------|-----------|-----------------|-----------------|
| ● antiAgA(seq1) | DVD-01 | antiVEGF(seq1) | antiAgA(seq2) | LS | 4.37E+04 | 4.52E-04 | 1.0E-08 | 0.8 | 2.22E+05 | 5.98E-05 | 2.7E-10 | 0.8 |
| ● antiAgA(seq2) | DVD-02 | antiVEGF(seq1) | antiAgA(seq1) | LL | 5.18E-04 | 7.33E-04 | 1.3E-08 | 0.5 | 3.57E+05 | 2.76E-03 | 7.7E-09 | 0.7 |
| ● antiAgA(seq3) | DVD-03 | antiAgA(seq2) | antiVEGF(seq1) | LL | 7.80E-04 | 8.65E-04 | 1.1E-08 | 0.9 | 5.77E+04 | 3.06E-05 | 5.3E-10 | 1.5 |
| ● antiAgA(seq4) | DVD-04 | antiAgA(seq3) | antiVEGF(seq1) | LL | 5.73E-08 | 2.62E-03 | 4.5E-09 | 0.9 | 4.53E+04 | 2.80E-05 | 6.2E-10 | 1.7 |
| ● antiAgA(seq5) | DVD-05 | antiAgA(seq4) | antiVEGF(seq1) | LL | 2.43E-05 | 1.62E-04 | 6.7E-10 | 0.6 | 6.55E+04 | 3.39E-05 | 5.1E-10 | 1.4 |
| ● antiAgA(seq6) | DVD-06 | antiAgA(seq5) | antiVEGF(seq1) | SL | 7.60E-04 | 7.82E-04 | 1.0E-08 | 0.9 | 5.31E+04 | 3.28E-05 | 6.2E-10 | 1.7 |
| ● antiAgA(seq7) | DVD-07 | antiAgA(seq6) | antiVEGF(seq1) | SL | 5.09E-08 | 2.83E-03 | 5.6E-09 | 0.7 | 6.08E+04 | 3.76E-05 | 6.3E-10 | 1.8 |
| ● antiAgA(seq8) | DVD-08 | antiAgA(seq7) | antiVEGF(seq1) | SL | 2.36E-05 | 1.64E-04 | 7.0E-10 | 0.7 | 5.71E+04 | 3.65E-05 | 6.7E-10 | 1.9 |
| ● antiAgA(seq9) | DVD-09 | antiAgA(seq8) | antiVEGF(seq1) | LS | 5.39E-06 | 2.77E-04 | 5.1E-10 | 0.7 | 2.88E+04 | 3.63E-05 | 1.2E-09 | 3.4 |
| ● antiAgA(seq10) | DVD-10 | antiAgA(seq9) | antiVEGF(seq1) | LS | 8.44E-04 | 1.01E-03 | 1.2E-09 | 0.9 | 2.09E+04 | 6.68E-05 | 3.2E-09 | 8.9 |
| ● antiAgA(seq11) | DVD-11 | antiAgA(seq10) | antiVEGF(seq1) | LS | 4.94E-05 | 2.37E-03 | 4.1E-09 | 0.9 | 2.37E+04 | 5.4E-05 | 2.6E-09 | 9.1 |
| ● antiAgA(seq12) | DVD-12 | antiAgA(seq11) | antiVEGF(seq1) | LS | 2.73E-05 | 1.66E-04 | 6.1E-10 | 0.6 | 2.97E+04 | 5.08E-05 | 1.7E-09 | 4.8 |
| ● antiAgA(seq13) | DVD-13 | antiAgA(seq12) | antiVEGF(seq1) | SS | 4.79E-05 | 2.72E-04 | 5.7E-10 | 0.8 | 9.35E+03 | 1.05E-04 | 1.1E-08 | 31.7 |
| ● antiAgA(seq14) | DVD-14 | antiAgA(seq13) | antiVEGF(seq1) | SS | 8.40E-05 | 8.98E-04 | 1.1E-08 | 0.8 | 2.98E+03 | 6.88E-05 | 2.3E-08 | 64.7 |
| ● antiAgA(seq15) | DVD-15 | antiAgA(seq14) | antiVEGF(seq1) | SS | 5.32E-05 | 2.87E-03 | 5.4E-09 | 0.7 | 1.09E+04 | 7.39E-05 | 6.8E-09 | 19.0 |

*Symbols shown are used to plot the kinetic binding constants in Figure 3B.*

**Accession code.** The X-ray crystallographic coordinates have been deposited in the Protein Data Bank with accession ID 4HJJ.

**Disclosure of Potential Conflicts of Interest**

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Author Contributions

R.E. purified the protein and prepared the complex. R.J. crystallized the complex. C.J. solved and analyzed the structure. J.G., and Y.L. completed sequence retrieval and design of VEGF-AgA DVD-IgTM’s, and E.D. performed the Biacore studies. C.J., T.G., R.J., and E.D. prepared the manuscript. T.G. leads the DVD-IgTM Initiative team.

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