

Reports

Molecular construction and optimization of anti-human IL-1 α / β dual variable domain immunoglobulin (DVD-IgTM) molecules

Chengbin Wu,* Hua Ying, Sahana Bose, Renee Miller, Limary Medina, Ling Santora and Tariq Ghayur

Biologics; Abbott Bioresearch Center; Worcester, MA USA

Abbreviations: mAb, monoclonal antibody; BsAb, bi-specific antibody; DVD-Ig, dual variable domain immunoglobulin; IL-1, interleukin-1; VH/VL, heavy/light variable domain; HC/LC, heavy/light chain; VD, variable domain; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; scFv, single chain Fv

Key words: DVD-Ig, dual variable domain immunoglobulin, interleukin-1, rheumatoid arthritis, variable domain, linker, antibody engineering, dual-specific antibody

Signal transduction through the interleukin-1 receptor (IL-1R) pathway mediates a strong pro-inflammatory response, which contributes to a number of human diseases such as rheumatoid arthritis. Within the IL-1 family, IL-1 α and IL-1 β are both agonistic ligands for IL-1R, whereas IL-1 receptor antagonist (IL-1ra) is an endogenous antagonist that binds to IL-1R, but does not signal. Therefore, the ideal therapeutic strategy would be blocking both IL-1 α and IL-1 β , but not IL-1ra. However, due to low sequence homology between the three members of the family, it has been exceedingly difficult to identify potent therapeutic agents, e.g., monoclonal antibodies (mAbs), that selectively recognize both IL-1 α and IL-1 β , but not IL-1ra. Currently, several anti-IL-1 therapeutic agents in clinical development either inhibit only IL-1 β (i.e., anti-IL-1 β mAb), or recognize all three ligands (i.e., anti-IL-1R mAb or IL-1R Trap). We have recently developed a novel dual variable domain immunoglobulin (or DVD-IgTM) technology that enables engineering the distinct specificities of two mAbs into a single functional, dual-specific, tetravalent IgG-like molecule. Based on this approach, we have developed anti-human IL-1 α / β DVD-IgTM molecules using several pairs of monoclonal antibodies with therapeutic potential, and present a case study for optimal design of a DVD-IgTM agent for a specific target pair combination.

Introduction

IL-1 α and IL-1 β , two pro-inflammatory cytokines of the IL-1 family, play a critical role in the pathogenesis of a variety of diseases.¹⁻³ Although these two cytokines share only about 20% primary amino acid identity, they have very similar overall tertiary

fold structures, bind to the same receptors and have almost identical biological functions.² In a disease setting, these two proteins may have redundant functions, therefore neutralizing the activities of both IL-1 α and IL-1 β may be required for optimal efficacy. In animal models, blocking functions of either IL-1 α or IL-1 β showed partial efficacy, whereas maximal efficacy was observed when both IL-1 α and IL-1 β functions were simultaneously blocked.⁴

An additional complexity in the IL-1 system is the existence of an endogenous inhibitor, IL-1ra, which binds IL-1R without signaling, and therefore negatively regulates IL-1 function. IL-1ra deficient mice spontaneously develop inflammatory disease,⁵ indicating that interference with the function of this naturally occurring antagonist may lead to undesired outcomes. The ideal therapeutic strategy would be to block both IL-1 α and IL-1 β , but not IL-1ra. However, due to low sequence homology between the three members of the family, it has not been technically feasible to identify potent therapeutic agents with this ability.

Monoclonal antibodies (mAb) have revolutionized treatment of a variety of autoimmune diseases and cancer, and hundreds of mAbs are in various stages of development.^{6,7} However, in most instances these mAb therapeutics benefit a subset of patients in a given indication. Human diseases are often complex, and both redundant and distinct disease mechanisms contribute to the final outcome of the disease. Combination mAb therapies are currently being pursued,⁸⁻¹⁰ but next generation biologic therapeutics that target two or more disease mechanisms could provide enhanced efficacy. Bispecific mAbs (bsAb) may offer several advantages over traditional IgG mAbs. For example, bsAbs, as multifunctional single agents, might be faster and less expensive to develop compared to combination therapy. BsAbs might also enhance efficacy and affect a larger percentage of patients. However, issues in manufacturing large quantities of clinical material, i.e., the drug substance, and pharmacokinetic properties of various bsAb formats have hampered their therapeutic development.¹¹⁻¹⁴

We have recently reported a novel technology for generating dual-specific biologic molecules, termed dual variable domain

*Correspondence to: Chengbin Wu; 100 Research Drive; Worcester, MA 01605 USA; Email: chengbin.wu@abbott.com

Submitted: 03/17/09; Accepted: 04/10/09

Previously published online as a mAbs E-publication:
<http://www.landesbioscience.com/journals/mabs/article/8755>

Figure 1. Schematic diagram of Dual Variable Domain (DVD)-Ig. (A) Generation DVD-Ig from two parental antibodies. The VH and VL of 13F5.G5 was directly fused to the N-terminus (without signal sequences) of the HC and LC of 3D12.E3, respectively, to generate HC and LC of DVD-Igs that were subsequently co-expressed in mammalian cells. (B) Specific constructs (1st set) generated include DVD1-Ig, DVD2-Ig and two chimeric mAbs from hybridoma clones 3D12.E3 (anti-IL-1 α) and 13F5.G5 (anti-IL-1 β).

immunoglobulin (DVD-IgTM).⁴ These DVD-IgTM molecules could be generated by combining the variable domains (VDs) of any two pre-existing mAbs, and readily manufactured from conventional mammalian cell production systems. In addition, they retained functional properties of both parental mAbs, and exhibited drug-like physicochemical and pharmacokinetic properties. Here we describe the development of DVD-IgTM molecules that target both IL-1 α and IL-1 β simultaneously, with a focus on optimization of molecular design that involves VD orientation, as well as linker lengths between the two VDs. The information presented here could enhance understanding of this class of molecules and benefit future effort in generating optimal DVD-IgTM therapeutic molecules.

Results

Construction and expression of anti-IL-1 α / β DVD-Ig proteins. Two antibodies directed against hIL-1 α (clone 3D12.E3) and hIL-1 β (clone 13F5.G5) were initially used for the construction of the first set of DVD-Ig molecules. Both parent mAbs are neutralizing antibodies that inhibit the biological functions of their respective antigen, and their chimeric format (murine variable domains with human constant regions) was used as a control because all DVD-Igs were also in the chimeric format, and so had human constant regions (Fig. 1A). DVD1-Ig was constructed by direct linkage of the VDs of heavy chain (HC) and light chain (LC) of 13F5.G5 to the N-terminus of the HC and LC (minus signal sequences) of 3D12.E3, respectively (Fig. 1B). In this orientation, the VDs of 13F5.G5 were in a position distal from the constant regions in primary sequences, whereas the VDs of 3D12.E3 were in the position proximal to the constant regions. The DVD2-Ig was constructed similarly, except that it has a linker between the two VDs in both the LC (the linker sequence is ADAAP) and the HC (the linker sequence is AKTTPP) (Fig. 1B). These linker sequences, selected from the N-termini of murine C κ and C λ 1, are natural extensions of the VDs and exhibit a flexible conformation without significant secondary structures based on the analysis of several Fab crystal structures. The HC and LC of each construct were subcloned into mammalian expression vectors and transiently expressed in COS and 293 cells. The cell culture media 72 hr-post transfection were quantified for human IgG by standard ELISA. Table 1 shows that the expression levels of DVD1-Ig and DVD2-Ig are comparable to that of the mAbs, indicating that the DVD-Ig can be expressed efficiently in mammalian cells.

Biochemical analysis of DVD-Ig. DVD-Igs and mAbs were purified by protein A chromatography. The purification yield (3–5 mg/L) was consistent with hIgG quantification of the expression medium for each protein. The composition and purity of the purified DVD-Igs and chimeric antibodies (Abs) were analyzed by

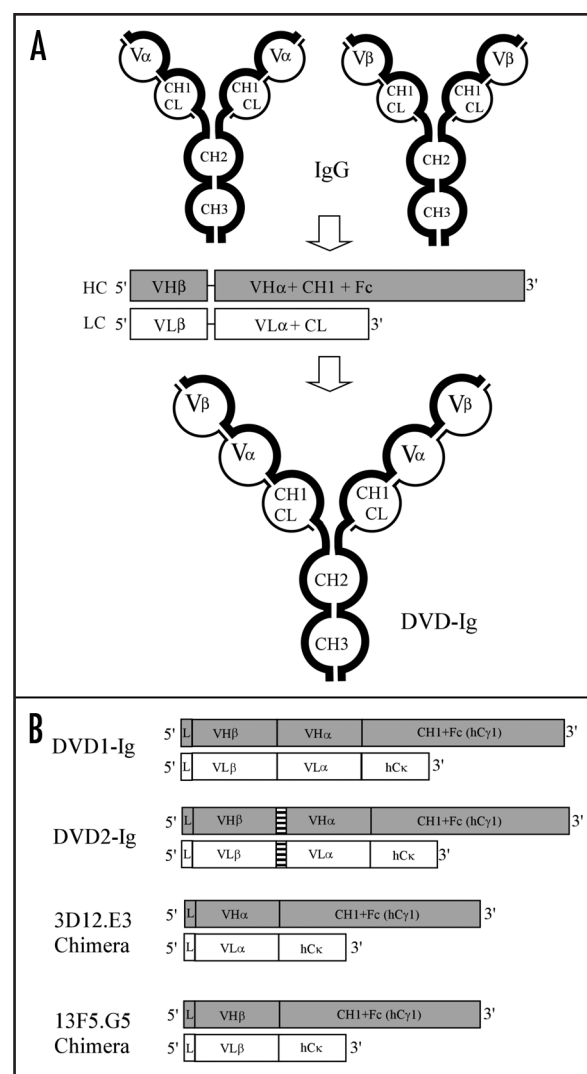


Table 1 Expression and molecular weight analysis of anti-hIL-1 α / β DVD-Ig molecules

Ig	Expression level (mg/L)		Molecular mass (Dalton)		
	COS	Freestyle 293	Light chain	Heavy chain	Full length
3D12.E3	2.80	3.90	23,696	49,914	147,220
13F5.G5	3.26	3.56	24,084	49,518	147,204
DVD1-Ig	3.00	3.30	35,797 (35,790)	64,380 (64,371)	200,346 (200,521)
DVD2-Ig	2.43	3.49	36,222 (36,220)	64,976 (64,973)	202,354 (202,573)

The molecular mass of the light chain, heavy chain, and full length of DVD1-Ig and DVD2-Ig determined experimentally by mass spectrometry are shown in parenthesis.

SDS-PAGE in both reducing and non-reducing conditions (Fig. 2A). In non-reducing condition, each of the four proteins migrated as a single band. The DVD-Ig proteins showed higher molecular weight (MW) than the mAbs, as expected. In reducing conditions,

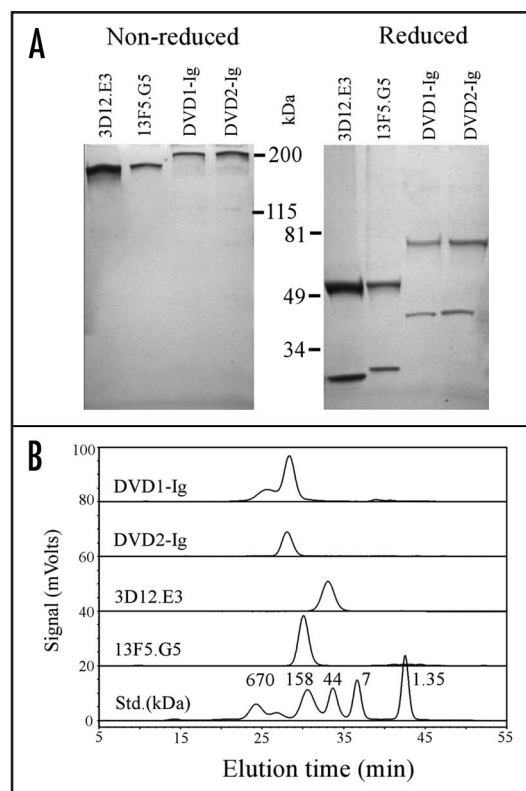


Figure 2. (A) SDS-PAGE analysis of DVD-Ig molecules. Purified chimeric mAbs 3D12.E3 and 13F5.G5, DVD1-Ig and DVD2-Ig were run on 10% SDS tris-glycine gel in non-reduced (left) and reduced (right) conditions. (B) Analysis of mAbs/DVD-Igs by size exclusion chromatography (SEC). Std: protein standards ranging from 1.35 to 670 kDa.

each of the four proteins yielded two bands, one HC and one LC. Again, the HC and LC of the DVD-Igs were larger in size than that of the chimeric Abs. SDS-PAGE showed that each DVD-Ig was expressed as a single species, and the HC and LC were efficiently paired to form an IgG-like molecule. In order to determine the precise MW of DVD-Ig, mass spectrometry was employed. As shown in Table 1, the experimentally determined molecular mass of each DVD-Ig, including the LC (~36 kDa), HC (~64 kDa), and the full-length protein (~200 kDa), was in good agreement with the predicted value of the respective sequence. To further study the physical properties of DVD-Ig in solution, size exclusion chromatography (SEC) was used to analyze each protein. As shown in Figure 2B, both mAbs and DVD2-Ig exhibited a single peak, demonstrating physical homogeneity as monomeric proteins. The 3D12.E3 mAb showed a smaller dynamic size compared to 13F5.G5 mAb, indicating that 3D12.E3 mAb adopted a more compact, globular shape. DVD1-Ig revealed a major peak, as well as a shoulder peak on the left, suggesting that a portion of DVD1-Ig was possibly in an aggregated form.

Stability study of DVD-Ig molecules. The long-term physical stability of an antibody or protein is critical in considering its potential use as a therapeutic agent. The physical stability of DVD-Ig was assessed by exposure of the molecules to multiple freeze-thaw cycles (between -80 and 25°C), as well as long-term storage at 4,

25 and 40°C for four weeks and eight weeks, followed by SEC analysis (Table 2). Both parental mAbs showed minor degrees of aggregation and fragmentation at 4°C, which were normal for regular research-grade IgG preparations. However, 3D12.E3 exhibited more thermal sensitivity after prolonged incubation, as assessed by the presence of more fragments at eight weeks, whereas 13F5.G5 was more prone to aggregation at 40°C. As shown in Figure 2B DVD1-Ig, revealed certain levels of aggregation on SEC after purification. DVD1-Ig also showed considerable aggregations under different conditions in the stability analysis; however, the percentage of aggregated form of DVD1-Ig did not increase during prolonged storage at higher temperatures. In fact, the aggregated form of DVD1-Ig appeared to decrease during storage at higher temperatures, which likely contributed to the increased percentage of monomeric fractions (Table 2). The percentages of the fragmented form of DVD1-Ig were similar to that of 3D12.E3. In contrast, DVD2-Ig showed exceptional stability. Neither aggregation nor fragmentation was detected at any significant levels in the conditions tested, and over 99% of DVD2-Ig was maintained as intact monomeric molecule.

Antigen-binding kinetics and dual binding activity of DVD-Ig. The binding kinetics of DVD-Igs and the mAbs were measured by surface plasmon resonance using a Biacore instrument (Table 3). The overall binding parameters of the two DVD-Igs to hIL-1 α were similar, with the affinities of the DVD-Igs being only 2–3 fold less than that of the parental mAb 3D12.E3. The binding affinity of DVD2-Ig to hIL-1 β was slightly less than the parental mAb 13F5.G5, but 3-fold higher than that of DVD1-Ig. The affinity profile of the two DVD-Igs to hIL-1 compared to that of the parental mAbs was also reflected in the evaluation of the stoichiometry of IL-1 binding. Both parental mAbs bound to IL-1 α and IL-1 β on Biacore with a stoichiometry of 1.6 and 1.7, respectively, suggesting bivalent binding. The stoichiometries of both DVD-Igs for hIL-1 α and hIL-1 β were similar to that of the two chimeric Abs, indicating that both DVD-Igs possessed bivalent binding capability to each antigen. To determine whether the DVD-Ig could bind two different antigens simultaneously as a tetravalent, dual-specific molecule, a multiple binding study was performed for DVD2-Ig using Biacore (Fig. 3). DVD2-Ig was first captured via a goat anti-human Fc antibody on the Biacore sensor chip, then the first antigen was injected and a binding signal observed. As the DVD2-Ig was saturated by the first antigen, the second antigen was subsequently injected and the second signal observed. This was done by first injecting IL-1 β , then IL-1 α (Fig. 3A) and, in the reverse order, IL-1 α was injected first, followed by IL-1 β (Fig. 3B) for DVD2-Ig. For both sequences, dual-binding activity was detected, and the same results were obtained for DVD1-Ig (data not shown). The results suggested that each DVD-Ig was able to bind both antigens simultaneously as a dual-specific, tetravalent molecule, and that the binding of the first antigen did not disrupt the binding of the second antigen, regardless which antigen bound first.

Analysis of the functional activity of DVD-Ig molecules. The biological function of the DVD-Ig molecules was analyzed by MRC-5 bioassay and their potencies to inhibit IL-1-induced IL-8 production were measured (Fig. 4, Table 3). Both DVD-Igs

Table 2 In vitro stability analysis of anti-hIL-1 α / β DVD-Ig proteins

	3D12.E3			13F5.G5			DVD1-Ig			DVD2-Ig		
	Agg	Ig	Frgm	Agg	Ig	Frgm	Agg	Ig	Frgm	Agg	Ig	Frgm
3xFreeze-Thaw	1.72	98.28	N.D.	13.0	87.0	N.D.	46.50	53.50	N.D.	N.D.	>99	N.D.
4°C 4 Wks	0.85	>99	N.D.	4.2	95.8	N.D.	42.43	56.63	0.94	N.D.	>99	N.D.
25°C 4 Wks	1.29	98.71	N.D.	N.D.	>99	N.D.	45.66	54.34	N.D.	N.D.	>99	N.D.
40°C 4 Wks	1.65	98.35	N.D.	20.3	78.1	1.6	36.70	59.42	3.88	N.D.	>99	N.D.
4°C 8 Wks	5.35	90.33	4.32	2.2	97.8	N.D.	38.18	56.91	4.91	N.D.	>99	N.D.
25°C 8 Wks	1.11	60.55	38.34	1.4	97.5	1.0	24.42	67.39	8.19	N.D.	>99	N.D.
40°C 8 Wks	4.74	81.47	13.79	34.6	65.4	N.D.	20.55	67.16	12.29	N.D.	>99	N.D.

The degree of aggregation and fragmentation are shown in percentage, whereas the percentage of Ig represents intact molecule. Agg, aggregates; Ig, intact monomeric Ig; Frgm, fragments. N.D. non-detectable.

were able to neutralize hIL-1 α (Fig. 4A) and hIL-1 β (Fig. 4B). Consistent with the binding affinity to hIL-1 α , the potencies of DVD1-Ig and DVD2-Ig against hIL-1 α were also similar, and were within 3-fold of that of the parental mAb (Fig. 4A, Table 3). Also in agreement with their binding affinities, the potency of DVD2-Ig to hIL-1 β was slightly less than that of the parental mAb 13F5.G5, but 3-fold higher than that of DVD1-Ig. However, the biological activity of DVD1-Ig might not have been precisely measured due to the presence of ~40% aggregate (Fig. 2B). Nevertheless, there was no significant decrease (within 5-fold) in the biological activities of DVD-Ig molecules compared to the original mAbs. To determine if DVD-Ig was able to inhibit IL-8 production in the presence of both IL-1 α and IL-1 β , equal amounts of hIL-1 α and hIL-1 β were added in the same culture system of MRC-5 bioassay (Fig. 4C). Both DVD1-Ig and DVD2-Ig were able to inhibit IL-8 synthesis by MRC-5 cells in the presence of both IL-1 α and IL-1 β , with potencies similar to that of the conventional assays where only one cytokine was present (Table 3). Again, the dual-inhibition potency of DVD2-Ig (1.2 nM) was slightly higher than that of DVD1-Ig (2.2 nM), probably due to their potency differences against hIL-1 β .

Optimization of DVD-Ig constructs. We next determined whether the DVD-Ig format could be generally applied to other pairs of mAbs, and the preferred VD orientation and linker length for an optimal anti-IL-1 α / β DVD-Ig construct. Two additional mAb pairs, 18F4.2C8 (anti-IL-1 α) and 1B12.4H4 (anti-IL-1 β), as well as 6H3.1A4 (anti-IL-1 α) and 6B12.4F6 (anti-IL-1 β) were used to construct a second set of DVD-Ig molecules. For each combination, two different domain orientations (α - β -Constant or β - α -Constant orientation) and two different linkers (short and long) were incorporated to evaluate their potential impact on the physical properties and biological functions of the resulting DVD-Ig molecules. Since extensive aggregation was observed in the DVD1-Ig that did not have a linker between the two VDs in both HC and LC, we speculated that a linker in the HC or LC might be necessary to achieve a physically monomeric, functionally dual-specific molecule, at least for this particular target combination. Therefore, a linker was included in all of the second set of constructs generated with these two new pairs of mAbs. Since we observed a minor decrease in antigen binding affinity in DVD2-Ig compared to the parental mAbs, we further extended the linker

Table 3 Functional characterization of anti-IL-1 α / β DVD-Ig molecules

	Antigen	k_{on} (M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	K_D (nM)	Stoichiometry	Potency IC ₅₀ (nM)
3D12.E3	hIL-1 α	4.12E+05	5.52E-04	1.34	1.6	0.70
DVD1-Ig	hIL-1 α	3.70E+04	1.05E-04	2.83	1.8	2.30
DVD2-Ig	hIL-1 α	7.35E+04	2.52E-04	3.42	2.0	2.90
13F5.G5	hIL-1 β	1.41E+06	6.54E-04	0.46	1.7	0.53
DVD1-Ig	hIL-1 β	6.09E+05	1.59E-03	2.60	1.5	3.10
DVD2-Ig	hIL-1 β	1.19E+06	9.50E-04	0.80	1.8	1.60

Affinity and stoichiometry were measured by Biacore; Potency (IC₅₀) was determined by MRC-5 bioassay.

(similar to the length used in DVD2-Ig) from 5 and 6-amino acids (aa) to 12 and 13-aa in length in VL and VH, respectively, to potentially add additional flexibility and reduce steric hindrance between the two VDs, and improve antigen binding affinity in the DVD-Ig molecules (Table 4). Since a lead molecule could potentially be identified from this effort, human Ig-derived linkers were used for the second set of DVD-Igs to avoid potential immunogenicity issues during future development.

The purified DVD-Ig molecules derived from 18F4.2C8 (anti-IL-1 α) and 1B12.4H4 (anti-IL-1 β) were named DVD3a-Ig, DVD4a-Ig, DVD3b-Ig and DVD4b-Ig depending on their domain orientation and linker size (Table 4). The MW of each DVD-Ig was confirmed by mass spectrometry. The profile of each of these DVD-Ig proteins by SEC exhibited a monomeric, intact, single species (Fig. 5). The dynamic sizes of DVD-Ig proteins were larger than those of the two parental mAbs, as expected (Fig. 5). Additional DVD-Igs derived from 6H3.1A4 (anti-IL-1 α) and 6B12.4F6 (anti-IL-1 β) also showed similar monomeric profiles by SEC (data not shown). It appeared that each of the second set of DVD-Ig molecules was efficiently expressed as single species, and purified to a homogeneous protein without noticeable aggregation.

The antigen binding affinity of the second set of DVD-Igs, as measured by Biacore, revealed interesting patterns. It is evident that all VDs in the distal position retained binding affinities (within 3-fold) of their respective parental mAbs. In contrast,

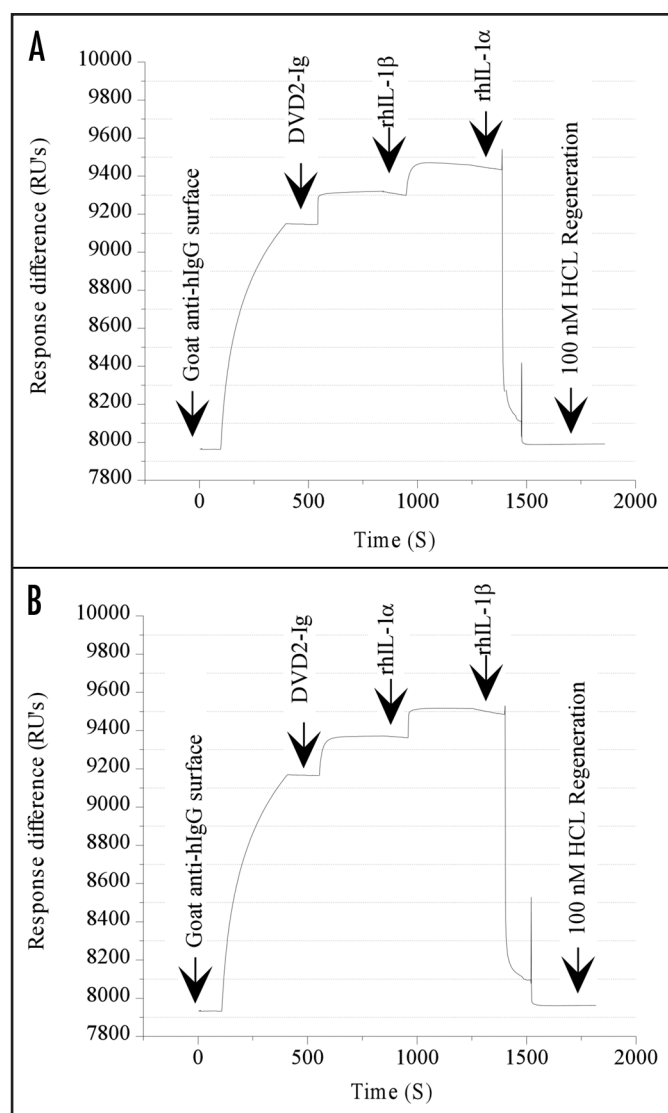


Figure 3. Binding mode analysis of DVD-Ig by Biacore. DVD2-Ig was immobilized by a goat anti-human Fc antibody on the sensor chip, and hIL-1 β was first injected and a binding signal observed, followed by the injection of hIL-1 α , which exhibited the second binding signal (A). In the reverse order, hIL-1 α was first injected followed by the injection of hIL-1 β (B), showing a similar result of dual-specific binding.

affinities of all VDs in proximal position were reduced substantially when a short linker was used, and their affinities were significantly improved when a long linker was employed. The neutralization potencies of some of these DVD-Igs were consistent with their respective binding affinities. For example, a marked decrease in binding affinity to IL-1 β was observed for DVD-3a-Ig, which subsequently did not show any inhibitory activity against IL-1 β in MRC-5 assay. As another example, DVD4b-Ig fully retained binding affinities and neutralization potencies against both antigens. However, some differences between affinity and potency existed for several of the DVD-Ig molecules. In the Biacore affinity measurements, the parental mAb and the DVD-Ig were captured on the chip by an anti-Fc IgG, thus limiting the full

motion/rotational flexibility of the captured molecule; Whereas, in a cell-based bioassay, both the antigen and the parental mAb or DVD-Ig were in solution, thereby allowing for full motion/rotational flexibility to both the ligand and the DVD-Ig molecule. Therefore, the neutralization potency data (Table 4) may better reflect the full functional characteristics of DVD-Ig molecules.

Discussion

Over the past two decades several bi-specific antibody formats have been described.¹¹⁻¹⁴ Although these formats can bind two different targets, significant hurdles in manufacturing, stability and pharmacokinetic properties have limited their progress through clinical development.¹¹⁻¹⁴ Antibodies are highly stable molecules with remarkable specificity for their targets. These physical and functional properties should be retained in any novel antibody-based therapeutic molecules. The overall organization of an antibody molecule, with distinct structural domains and peptide linkages, provides critical flexibility for antigen binding that may also be important for the overall physical stability of the molecule.¹⁵ In addition, crystal structures of mAbs (e.g., Fabs) have clearly demonstrated that the complementarity-determining regions (CDR) that interact with antigen are exposed,¹⁶ suggesting that any structures in close proximity may disrupt functions of the CDRs. This notion was revisited when we recently reported a novel model of dual-specific biologic molecule, DVD-Ig,⁴ which can be readily generated by combining the VDs of any two mAbs. Such DVD-Ig molecules retained binding activities of the two parental mAbs, expressed well in mammalian cells, could be purified efficiently as homogeneous protein, and displayed drug-like properties with circulating serum $t_{1/2}$ similar to that of mAbs.

As reported here, we further explored the contributions of individual mAb VDs, including their orientations and linker lengths, to DVD-Ig construction by studying multiple mAb pairs to anti-human IL-1 α and anti-human IL-1 β . We also optimized desired features of these molecules, including expression in mammalian cells, in-vitro stability and preservation of binding properties.

To delineate specific structural and functional requirements for a particular DVD-Ig, we generated and evaluated two sets of anti-IL-1 α / β DVD-Ig molecules. In the first set of DVD-Ig (DVD1-Ig and DVD2-Ig), DVD1-Ig had two VDs in each LC and HC linked directly without a spacer in between, whereas in DVD2-Ig a short linker of 5–6 aa was used to fuse the two VDs together. Stoichiometric analysis of the two purified DVD-Ig revealed that both DVD1-Ig and DVD2-Ig were capable of binding two IL-1 α and two IL-1 β molecules. Although both DVD-Igs effectively retained the biological activities and potencies of the parent mAbs, DVD2-Ig displayed slightly better anti-IL-1 β activities than did DVD1-Ig, probably because the linkers between the two VDs in DVD2-Ig allowed more flexibility and less steric hindrance. Surprisingly, anti-IL-1 α activity was also well-maintained in both DVD-Igs, even though its VH/VL was in a proximal position within the DVD-Ig molecules. However, the functional activity of DVD1-Ig might have not been precisely determined due to significant high MW aggregation, which was not observed in DVD2-Ig. The aggregated form of DVD1-Ig was about 40%, and the

biological activity of the aggregated DVD1-Ig was unknown. It would be reasonable to assume that the observed activity of DVD1-Ig was due primarily to the 60% monomeric form. While the 40% aggregate has a significant impact on the physicochemical properties of DVD1-Ig, it may affect the potency measurement by less than 2-fold. Nevertheless, this study illustrates the importance of linkers between the two VDs. The lack of a peptide spacer between the two VDs may cause certain steric hindrance, possibly leading to misfolding and aggregation. However, the aggregated form of DVD1-Ig seemed to be partially reversible in response to increased temperature, and did not aggravate during repeated freeze-thaw or prolonged storage. In contrast, the DVD2-Ig molecule was remarkably stable in comparison to the two parental mAbs, and remained as >99% intact, monomeric protein even after storage at 40°C for eight weeks. It was also surprising that DVD2-Ig seemed to be more stable than the parental mAbs. A possible explanation is that the additional VH/VL domains at the distal position might strengthen the HC/LC association, rendering the molecule more stable. Future in-depth thermal stability studies using differential scattering colorimetry might address this issue. Collectively, these data demonstrated that the absence of a peptide linkage between the two VDs had minimal impact on antigen binding properties of the two VDs, but drastically impacted physical properties of DVD1-Ig, at least for this particular pair of mAbs (3D12.E3 and 13F5.G5). Further studies will evaluate the role of linkers in influencing physical property in general, as well as in vivo stability of DVD-Ig molecules.

To determine whether DVD-Ig design can be applied to other mAb pairs, we used two additional pairs of anti-IL-1 α (18F4.2C8 and 6H3.1A4), anti-IL-1 β (1B12.4H4 and 6B12.4F6) mAbs to construct several different DVD-Igs. Based on the initial data showing the importance of a linker, DVD-Ig molecules were constructed with either a short or a long linker between the two VDs. In addition, we also tested whether different domain orientations (i.e., placing each VD either in the proximal or the distal position) could result in improved functional properties of a DVD-Ig molecule. The monomeric profiles of the second set of DVD-Igs (DVD3a/b-Ig to DVD6a/b-Ig) confirmed the critical role of linkers between the two VDs. Interestingly, DVD-Igs with long linkers (DVD4a-Ig and DVD4b-Ig) exhibited a lightly smaller dynamic size compared to their short-linker counterpart (DVD3a-Ig and DVD3b-Ig), suggesting that the DVD-Igs with long linkers might adopt a more compact structural fold. The long linkers potentially allowed an inward folding of the distal VH/VL domains (elbow bending at the linker region), therefore reducing the overall dynamic size of the full-length protein. The functional analysis of the second set of DVD-Igs was quite revealing, suggesting a certain pattern that may aid in better design of the DVD-Ig molecule for IL-1 α / β . A key observation was that VDs from all mAbs retained biological functions (potency within 2–10 fold) of their respective parental mAbs when placed at the distal position in a DVD-Ig molecule, likely owing to lack of steric hindrance and full exposure of the CDRs. This is consistent with our previous observations with distinct DVD-Igs against other target pairs.⁴ In addition, the VDs from both anti-IL-1 α mAbs,

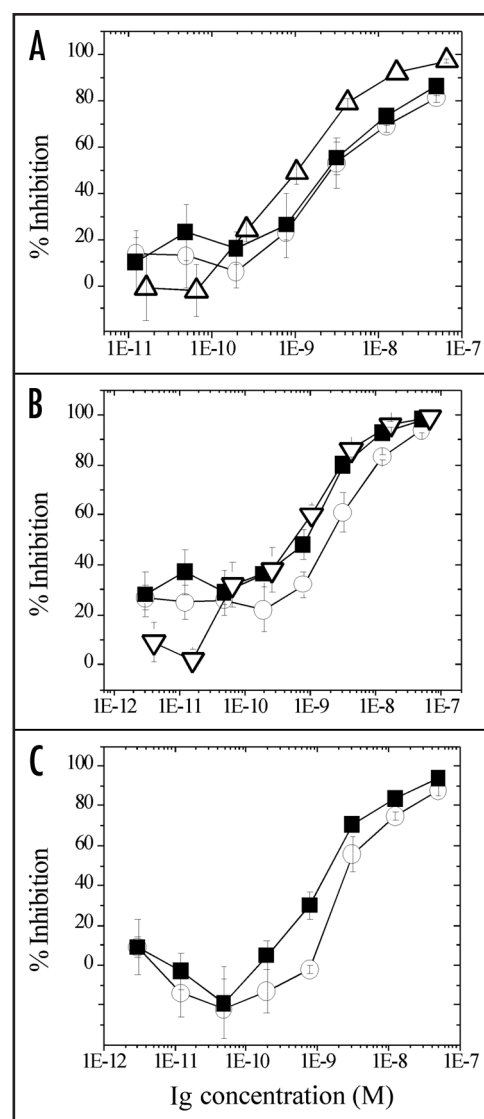


Figure 4. Inhibition of both IL-1 α and IL-1 β biological activities by DVD-Ig. DVD1-Ig (○) and DVD2-Ig (■) were tested in MRC-5 bioassay to determine their neutralization potency against hIL-1 α (A) and hIL-1 β (B), in comparison to 3D12.E3 (△) and 13F5.G5 (▽) chimeric mAbs. DVD1-Ig and DVD2-Ig were also able to inhibit IL-8 production in the presence of both IL-1 α and IL-1 β in the same cell culture system (C).

when placed on the proximal position, retain substantial potency of parental mAbs, particularly with a long linker (potencies within 5-fold of parental mAb), suggesting that the VDs of these parental mAbs are more “compatible” with the proximal position within the DVD-Ig. In contrast, VDs from both anti-IL-1 β mAbs placed in the proximal position showed substantially reduced potency (>10-fold less than that of parental mAbs) regardless of linker length. This did not appear to be an epitope-dependent phenomenon since the anti-IL-1 β mAbs targeted different epitopes as assessed by sandwich ELISA (data not shown), nor was it due to the dynamic size of the targets because IL-1 α and IL-1 β are similar in size and MW. For DVD-Igs with an α - β -constant orientation (DVD3a-Ig, DVD4a-Ig, DVD5a-Ig, DVD6a-Ig), increasing

Table 4 Domain orientation and linker optimization for anti-IL-1 α / β DVD-Ig molecules

Ig	Orientation	Linker	Affinity (K_D) nM		Potency (IC_{50}) nM	
			hIL-1 α	hIL-1 β	hIL-1 α	hIL-1 β
18F4.2C8			0.60		0.33	
1B12.4H4				0.26		0.60
DVD3a-Ig	α - β -C	short	0.84	63.7	0.75	NA
DVD4a-Ig	α - β -C	long	0.70	0.93	0.35	10.0
DVD3b-Ig	β - α -C	short	1.24	0.19	0.70	0.40
DVD4b-Ig	β - α -C	long	0.56	0.13	0.35	0.50
6H3.1A4			0.35		0.24	
6B12.4F6				0.55		0.40
DVD5a-Ig	α - β -C	short	0.51	12.5	2.60	19.0
DVD6a-Ig	α - β -C	long	1.06	2.09	2.30	70.0
DVD5b-Ig	β - α -C	short	13.2	0.67	3.30	0.25
DVD6b-Ig	β - α -C	long	0.82	0.70	1.00	0.75

Affinity (K_D) was measured by Biacore; Potency (IC_{50}) was determined by MRC-5 bioassay. NA: no activity was detected.

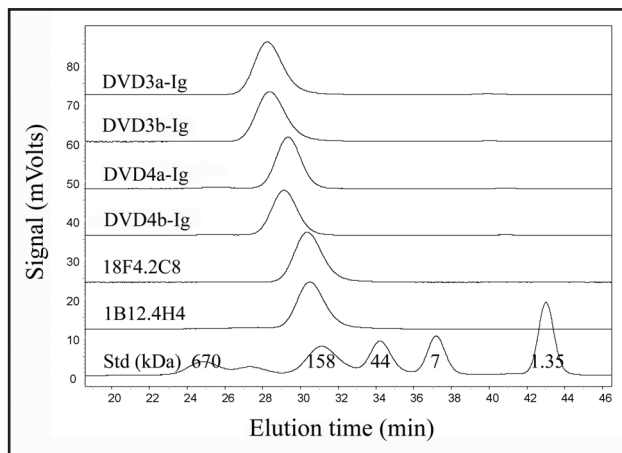


Figure 5. Monomeric profiling of the second set of mAbs/DVD-Igs by size exclusion chromatography. Std: protein standards ranging from 1.35 to 670 kDa.

linker length was not sufficient to significantly regain activity of the anti-IL-1 β mAbs (Table 4). Collectively, these observations suggest that, in some instances, increasing linker length may not be sufficient to fully retain the proximal domain function of the DVD-Ig. Interestingly, when anti-IL-1 α VD were placed at the proximal position, a number of DVD-Igs displayed desired properties due to the fact that functions of both parental mAbs were well-maintained. For example, DVD4b-Ig fully retained binding affinities and neutralization potencies against both antigens. This observation highlights the importance of domain and linker optimization in developing a DVD-Ig molecule. Many additional factors, such as target size and shape, epitopes recognized by the parental mAbs, and intrinsic properties of VDs may all influence the final functional and physicochemical features of the DVD-Ig

molecule. The intrinsic properties of a VD may include its surface charge, structural fold (i.e., canonical structure),¹⁷ dynamic size and shape and CDR loop sizes. A combination of these factors may determine the compatibility of a VD in a DVD-Ig construct, particularly in a proximal position, and so each DVD-Ig must be treated as a unique entity, with specific structural and functional properties for a particular mAb combination. As the compatibility of a VD for DVD-Ig may not be readily pre-determined, it will be ideal to test a panel of diverse mAb pairs in order to construct an optimal DVD-Ig molecule.

In this study, we have described DVD-Ig molecules that can block functions of both human IL-1 α and human IL-1 β . These IL-1 agonists, along with the IL-1 receptor antagonist IL-1ra, are key members of the IL-1 protein family.² Although these three proteins show only about 20% primary amino acid identity, they have similar tertiary structures, and bind to the same receptors with similar affinities. IL-1 α and IL-1 β display almost identical agonist functions. As a receptor antagonist, IL-1ra binds the receptor, but does not transduce signal. In preclinical models of rheumatoid arthritis, we and others have clearly demonstrated that blocking either IL-1 α or IL-1 β results in only partial efficacy, whereas neutralizing functions of both IL-1 α and IL-1 β simultaneously completely prevents disease progression.^{4,18,19} In addition, we (unpublished observations) and others have observed that mice lacking IL-1ra spontaneously develop autoimmune disease-like syndromes, including rheumatoid arthritis (RA)-like disease of the joints.⁵ Collectively, these observations suggest that maximum efficacy in chronic autoimmune diseases might be achieved by a therapeutic agent that blocks the functions of both IL-1 α and IL-1 β , but not IL-1ra. Several different approaches are currently being pursued to modulate IL-1 activity in a variety of disease settings. A recombinant IL-1 receptor antagonist (anakinra) that competes with both IL-1 α and IL-1 β for IL-1R binding has shown efficacy in several diseases, but its efficacy is not as good as expected in RA, and less than anti-TNF biologics, possibly due to its short half-life.²⁰ Other biologics therapeutic approaches in development do not specifically block functions of both IL-1 α and IL-1 β simultaneously. The IL-1 Trap (rilonacept), an IL-1R-Fc fusion protein, binds IL-1 α , IL-1 β and IL-1ra, and therefore will block functions of all three IL-1 family members. This agent did not achieve significant efficacy in RA clinical trials.²¹ Canakinumab, an anti-IL-1 β mAb now in clinical trials, blocks the function of only IL-1 β .²² An anti-IL-1R mAb (AMG 108) that is also in clinical development blocks binding of IL-1 α , IL-1 β and IL-1ra to the IL-1R. It also potentially interferes with soluble decoy receptors that mechanistically benefit in downregulating IL-1 signal. The DVD-Ig format, as described here, offers an opportunity to specifically target IL-1 α and IL-1 β , but not IL-1ra.

With the US Food and Drug Administration's approval of over 20 therapeutic mAbs, and with over 200 mAbs in various stages of development, the molecules are now considered a critical component of the pipelines of major pharmaceutical companies.^{6,7} However, the growing body of clinical data on therapeutic mAbs suggests that there is room for improvement, and the next generation of antibody (or antibody-like) drugs should have additional

features for better efficacy and greater patient response rate. Desired features for next generation molecules include specific targeting of two or more disease mechanisms simultaneously; improved ability to recruit immune cells or other enhanced effector functions for cancer treatment; appropriate pharmacokinetic properties and ease of manufacturing. These features can now be incorporated in DVD-Ig molecules. Like TNF, IL-1 is widely considered to be a major pro-inflammatory cytokine. However, in contrast to anti-TNF biologics, the therapeutic potential of IL-1 inhibitors have not been fully realized in the clinic, potentially due to difficulties in targeting the right components within this complex multi-ligand system. With the development of an anti-IL-1 α/β specific agent, the DVD-Ig therapeutic molecule will enable clinical validation of this important pathway in several human indications.

Material and Methods

Construction, expression and purification of anti-IL-1 α/β DVD-Ig proteins. The DVD-Ig constructs were generated as described previously.⁴ In principle, DVD-Ig is designed as an IgG-like molecule except that each LC and HC of a DVD-Ig molecule has two VDs (Fig. 1A), instead of one VD. To generate the first set of anti-IL-1 α/β DVD-Ig molecules, two parent mAbs, anti-hIL-1 α (clone 3D12.E3) and anti-hIL-1 β (clone 13F5.G5), were used. The VL/VH cDNAs of these two hybridoma clones were isolated by RT-PCR using the mouse Ig Primer Kit (Novagen, Madison, WI). They were then converted into chimeric antibodies (with human constant regions) to confirm activity and potency, and then used for subsequent studies as positive controls for the DVD-Igs derived from them. To generate DVD1-Ig, the VH and VL of 13F5.G5 were directly fused to the N-terminus (without signal sequences) of the HC and LC of 3D12.E3, respectively (Fig. 1A). The DVD2-Ig was constructed similarly, except that a linker between the two VDs in both the LC (the linker sequence is ADAAP) and the HC (the linker sequence is AKTTPP) was included (Fig. 1B). These linker sequences were selected from the N-termini of murine C κ and CH1 sequences. The HC and LC of each construct was subcloned into pcDNA3.1 TOPO and pEF6 TOPO vectors (Invitrogen Inc.), respectively, and sequenced to ensure accuracy. The plasmids encoding the HC and LC of each construct were transiently expressed in COS cells, as well as human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA) and purified using protein A chromatography (Pierce, Rockford, IL) according to manufacturer's instructions, and analyzed by SDS-PAGE and quantitated by A280 and BCA (Pierce, Rockford, IL). A second set of anti-IL-1 α/β DVD-Ig molecules (Table 4) was also generated with two different additional pairs of mAbs: mAbs 18F4.2C8 (anti-IL-1 α) and 1B12.4H4 (anti-IL-1 β) were used to construct DVD3a-Ig, DVD4a-Ig, DVD3b-Ig and DVD4b-Ig. DVD3a-Ig and DVD4a-Ig were in α - β -C (α - β -constant domain) orientation, with a short and long linker, respectively. DVD3b-Ig and DVD4b-Ig were in β - α -C orientation, with a short and long linker, respectively. Antibodies 6H3.1A4 (anti-IL-1 α) and 6B12.4F6 (anti-IL-1 β) were used to construct DVD5a-Ig, DVD6a-Ig, DVD5b-Ig and DVD6b-Ig. DVD5a-Ig and DVD6a-Ig were in α - β -C

orientation, with a short and long linker, respectively. DVD5b-Ig and DVD6b-Ig were in β - α -C orientation, with a short and long linker, respectively. None of the anti-hIL-1 α mAbs used in this report recognize hIL-1 β , and the anti-hIL-1 β mAbs employed in this study do not recognize hIL-1 α . The linker sequences, derived from the N-terminal sequence of human C κ or CH1 domain, are as follows: Short linker: LC: TVAAP; HC: ASTKGP. Long linker: LC: TVAAPSVFIFPP; HC: ASTKGPSVFPLAP. All HC and LC constructs were subcloned into mammalian expression vectors and produced and purified as described above.

Mass spectrometry analysis of DVD-Ig. For measuring molecular weight (MW) of LC and HC of DVD-Ig molecules, 10 μ L of DVD-Ig (0.8 μ g/ μ L) was reduced by 1.0 M DTT solution (5 μ L). A PLRP-S, 8 μ , 4,000 Å, and 1 x 150 mm protein column (Michrom BioResource, Auburn, MA) was used to separate heavy and LCs of DVD-Ig. Agilent HP1100 Capillary HPLC (Agilent Technologies Inc., Palo Alto, CA) was used with the mass spectrometer QSTAR (Applied Biosystems, Foster City, CA). The HPLC flow rate was 50 μ L/min, and the sample injection volume was 8.0 μ L. To determine the MW of full length DVD-Ig, a Protein MicroTrap cartridge (Michrom BioResource, Auburn, MA) was used for desalting the sample. The HPLC gradient was: 5%B for 5 minutes; 5%B to 95%B in 1 minute, and from 95%B to 5%B in another 4 minutes. All MS raw data were analyzed using the Analyst QS software (Applied Biosystems).

Size exclusion chromatography. Purified DVD-Ig proteins and chimeric mAbs, in PBS, were applied on a Superose 6 10/300 G₂, 300 x 10 mm column (Amersham Bioscience, Piscataway, NJ). An HPLC instrument, Model 10A (Shimadzu, Columbia, MD) was used for SEC. All proteins were determined using UV detection at 280 nm and 214 nm. The elution was isocratic at a flow rate of 0.5 mL/min. For the stability study, samples in the concentration range of 0.2–0.4 mg/ml in PBS underwent three freeze-thaw cycles between -80 and 25°C, or were incubated at 4, 25 or 40°C, for 4 weeks and 8 weeks, followed by SEC analysis.

Binding affinity measurements. The kinetics of DVD-Ig binding to hIL1 α/β was determined by surface plasmon resonance-based measurements with a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden), as described previously.⁴ Briefly, 5,000 RU of goat anti-human IgG Fc γ fragment specific polyclonal antibody (Pierce Biotechnology Inc., Rockford, IL) diluted in 10 mM sodium acetate (pH 4.5) was directly immobilized across a CM5 research grade biosensor chip using a standard amine coupling kit according to manufacturer's instructions. Purified mAb or DVD-Ig samples (0.1–1 μ g/mL) were diluted in HEPES-buffered saline for capture across goat anti-human IgG Fc specific reaction surfaces and injected over reaction matrices at a flow rate of 5 μ L/min. No obvious dissociation between anti-hFc and DVD-Ig (or mAbs) was observed during a pre-run with buffer only. After pre-run with buffer, aliquots of rhIL1 α/β samples were injected at ten different antigen concentrations ranging from 1.25 to 1,000 nM under a continuous flow rate of 25 μ L/min to determine the association and dissociation rate constants, k_{on} (M⁻¹s⁻¹) and k_{off} (s⁻¹). Any background signals from the buffer pre-run were subtracted from the overall binding signal. The equilibrium dissociation constant (M)

of the reaction was then calculated from the kinetic rate constants by the following formula: $K_D = k_{off}/k_{on}$. The apparent stoichiometry of the captured DVD-Ig-rhIL1 α / β complex was calculated under saturating binding conditions (steady-state equilibrium) using the following formula:

$$\text{Stoichiometry} = [\text{rhIL1}\alpha/\beta \text{ response (RU)}]/[\text{DVD-Ig response (RU)}] \times [\text{DVD-Ig M.W.}]/[\text{rhIL1}\alpha/\beta \text{ M.W.}]$$

In order to determine if DVD-Ig bound two targets simultaneously, multiple binding studies were performed on Biacore. Briefly, DVD-Ig was first captured via a goat anti-human Fc γ antibody on the Biacore sensor chip. The first antigen was injected and a binding signal observed. As the DVD-Ig was saturated by the first antigen, the second antigen was then injected and the second signal observed. This was done either by first injecting IL-1 α and then IL-1 β , or by first injecting IL-1 β followed by IL-1 α . In either sequence, a dual-binding activity was detected indicating that DVD-Ig was able to bind both antigens simultaneously as a dual-specific tetravalent molecule.

Functional assay. The potency of each mAb and DVD-Ig was measured by MRC-5 bioassay. The MRC-5 cell line is a human lung fibroblast cell line that produces IL-8 in response to human IL-1 α and IL-1 β in a concentration-dependent manner.²³ MRC-5 cells were originally obtained from ATCC and cultured in our laboratory in 10% FBS complete MEM and grown at 37°C in a 5% CO₂ incubator. To determine neutralizing potencies of the mAbs and DVD-Igs against human IL-1 α or IL-1 β , 50 μ l of mAb/DVD-Ig (1E-7 to 1E-11 M) in MEM/10% FBS was added to a 96 well plate and pre-incubated with 50 μ l of hIL-1 α or hIL-1 β (200 pg/ml) for 1 hr at 37°C, 5% CO₂. MRC-5 cells at a concentration of 10⁵/mL were then added (100 μ l) to all wells, and the plates were incubated overnight at 37°C in a 5% CO₂ incubator. To determine dual-inhibition activity of DVD-Ig molecules, both IL-1 α and IL-1 β (200 pg/mL each) were added to the cells. The supernatants were harvested, and human IL-8 production measured by standard ELISA (R&D Systems, Minneapolis, MN). Antibody potency was determined by its ability to inhibit IL-8 production.

References

1. Apte RN, Voronov E. Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? *Immunol Rev* 2008; 222:222-41.
2. Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clin Exp Immunol* 2007; 149:217-25.
3. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 2008; 118:3537-45.
4. Wu C, Ying H, Grinnell C, Bryant S, Miller R, Clabbers A, et al. Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin. *Nat Biotechnol* 2007; 25:1290-7.
5. Nicklin MJ, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. *J Exp Med* 2000; 191:303-12.
6. Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. Monoclonal antibody successes in the clinic. *Nat Biotechnol* 2005; 23:1073-8.
7. Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin Pharmacol Ther* 2008; 84:548-58.
8. Larbouret C, Robert B, Navarro-Teulon I, Thezenas S, Ladjemi MZ, Morisseau S, et al. In vivo therapeutic synergism of anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies against pancreatic carcinomas. *Clin Cancer Res* 2007; 13:3356-62.
9. Strauss SJ, Morschhauser F, Rech J, Repp R, Solal-Celigny P, Zinzani PL, et al. Multicenter phase II trial of immunotherapy with the humanized anti-CD22 antibody, epratuzumab, in combination with rituximab, in refractory or recurrent non-Hodgkin's lymphoma. *J Clin Oncol* 2006; 24:3880-6.
10. Way J, Super M. The potential for cancer combination therapy with multi-targeted, single-protein pharmaceuticals. *Expert Opinion on Drug Discovery* 2008; 3:147-52.
11. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 2005; 23:1126-36.
12. Marvin JS, Zhu Z. Recombinant approaches to IgG-like bispecific antibodies. *Acta Pharmacol Sin* 2005; 26:649-58.
13. Morrison SL. Two heads are better than one. *Nat Biotechnol* 2007; 25:1233-4.
14. Muller D, Kontermann RE. Recombinant bispecific antibodies for cellular cancer immunotherapy. *Curr Opin Mol Ther* 2007; 9:319-26.
15. Sandin S, Ofverstedt LG, Wikstrom AC, Wrangé O, Skoglund U. Structure and flexibility of individual immunoglobulin G molecules in solution. *Structure* 2004; 12:409-15.
16. Evans EJ, Esnouf RM, Manso-Sancho R, Gilbert RJ, James JR, Yu C, et al. Crystal structure of a soluble CD28-Fab complex. *Nat Immunol* 2005; 6:271-9.
17. Chothia C, Lesk AM. Canonical structures for the hypervariable regions of immunoglobulins. *J Mol Biol* 1987; 196:901-17.
18. Joosten LA, Helsen MM, Saxne T, van De Loo FA, Heinegard D, van Den Berg WB. IL-1 α blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF α blockade only ameliorates joint inflammation. *J Immunol* 1999; 163:5049-55.
19. van den Berg WB, Joosten LA, Helsen M, van de Loo FA. Amelioration of established murine collagen-induced arthritis with anti-IL-1 treatment. *Clin Exp Immunol* 1994; 95:237-43.
20. Cohen S, Hurd E, Cush J, Schiff M, Weinblatt ME, Moreland LW, et al. Treatment of rheumatoid arthritis with anakinra, a recombinant human interleukin-1 receptor antagonist, in combination with methotrexate: results of a twenty-four-week, multicenter, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 2002; 46:614-24.
21. Burger D, Dayer JM, Palmer G, Gabay C. Is IL-1 a good therapeutic target in the treatment of arthritis? *Best Pract Res Clin Rheumatol* 2006; 20:879-96.
22. Church LD, McDermott MF. Canakinumab, a fully-human mAb against IL-1 β for the potential treatment of inflammatory disorders. *Curr Opin Mol Ther* 2009; 11:81-9.
23. Dinarello CA, Muegge K, Durum SK. Measurement of soluble and membrane-bound interleukin 1 using a fibroblast bioassay. *Curr Protoc Immunol* 2000; 6:1.