

Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin

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For complex diseases in which multiple mediators contribute to overall disease pathogenesis by distinct or redundant mechanisms, simultaneous blockade of multiple targets may yield better therapeutic efficacy than inhibition of a single target. However, developing two separate monoclonal antibodies for clinical use as combination therapy is impractical, owing to regulatory hurdles and cost. Multi-specific, antibody-based molecules have been investigated; however, their therapeutic use has been hampered by poor pharmacokinetics, stability and manufacturing feasibility. Here, we describe a generally applicable model of a dual-specific, tetravalent immunoglobulin G (IgG)-like molecule—termed dual-variable-domain immunoglobulin (DVD-Ig)—that can be engineered from any two monoclonal antibodies while preserving activities of the parental antibodies. This molecule can be efficiently produced from mammalian cells and exhibits good physicochemical and pharmacokinetic properties. Preclinical studies of a DVD-Ig protein in an animal disease model demonstrate its potential for therapeutic application in human diseases.

Specifically targeting a single molecule with monoclonal antibody (mAb) therapy has emerged as an important therapeutic modality for human diseases. In most biologic systems, however, multiple disease mediators are involved in pathogenesis by either playing redundant roles in inducing the same signaling cascade, such as interleukin-1 α (IL-1 α) and IL-1 β ¹, or stimulating two independent cellular pathways in a disease process, such as tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) in rheumatoid arthritis². Therefore, simultaneous blockade of these effector molecules is likely to provide better clinical efficacy and/or reach a broader patient population than inhibition of a single target. The utility of combining two mAbs^{1,3} has already been demonstrated in preclinical models, and such an approach is also being investigated in the clinic⁴. However, the option of using approved mAbs for combination therapy is limited, owing to the small number of therapeutic mAbs currently on the market. In addition, the preclinical safety and efficacy of the combination of the marketed mAbs have probably not been adequately addressed. Therefore, testing these combinations directly in humans may have safety risks, and additional preclinical and clinical studies may be necessary. A different approach is to develop pairs of mAbs as combination therapies, which is costly and subject to substantial regulatory hurdles. Therefore, additional approaches to address multi-specificity issues for antibody-based therapeutics are needed.

During the last decade, genetic engineering has led to the development and optimization of various multi-specific antibody formats^{5–7}. Although multi-specificity has been achieved at the molecular level, two major hurdles still remain: manufacturing efficiency and *in vivo*

drug-like properties (pharmacokinetics, efficacy), the absence of which has hampered clinical use of these molecules as therapeutics, particularly for prolonged treatment of chronic diseases. Herein, we describe the design of a DVD-Ig, which can be engineered from any two mAbs of distinct specificities. The DVD-Ig protein is an IgG-like molecule that can be efficiently produced by conventional mammalian expression systems as a single species for easy manufacturing and purification, while maintaining the affinities and potencies of the two parental mAbs. In addition, it is highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties.

RESULTS

Engineering, expression and characterization

We engineered a human DVD-Ig protein using a human anti-IL-12 (p40) mAb 1D4.1 (ref. 8), and a human anti-IL-18 mAb ABT-325 (ref. 9), both of which are human IgG1s with κ light chains. To construct the DVD-Ig molecule, the V_{κ} domains of the two mAbs were fused in tandem by a short linker (TVAAP) with the variable domain of a 1D4.1 light (V_L) chain at the N terminus, followed by ABT-325 V_L and C_{κ} to form the DVD-Ig protein light chain. Similarly, the variable regions of the heavy (V_H) chain of the two mAbs were fused in tandem by a short linker (ASTKGP) with 1D4.1 V_H at the N terminus, followed by ABT-325 V_H and the heavy chain constant domains to form the 1D4.1-325 DVD-Ig protein heavy chain (Fig. 1a). Different DVD-Ig molecules are named as X-Y DVD-Ig protein, where X is the clone number (or name) of the mAb whose V_H/V_L are on the N termini of the heavy chain/light chain, respectively

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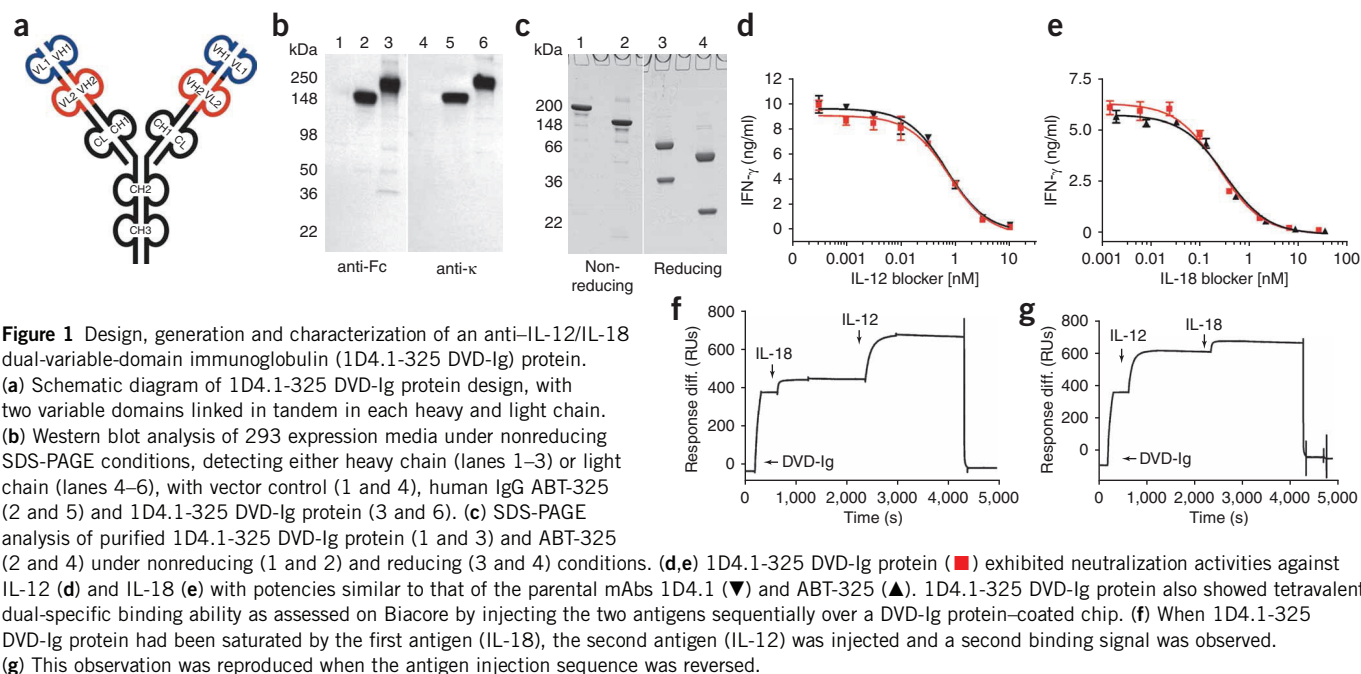


Figure 1 Design, generation and characterization of an anti-IL-12/IL-18 dual-variable-domain immunoglobulin (1D4.1-325 DVD-Ig) protein.

(a) Schematic diagram of 1D4.1-325 DVD-Ig protein design, with two variable domains linked in tandem in each heavy and light chain. (b) Western blot analysis of 293 expression media under nonreducing SDS-PAGE conditions, detecting either heavy chain (lanes 1–3) or light chain (lanes 4–6), with vector control (1 and 4), human IgG ABT-325 (2 and 5) and 1D4.1-325 DVD-Ig protein (3 and 6). (c) SDS-PAGE analysis of purified 1D4.1-325 DVD-Ig protein (1 and 3) and ABT-325 (2 and 4) under nonreducing (1 and 2) and reducing (3 and 4) conditions. (d,e) 1D4.1-325 DVD-Ig protein (■) exhibited neutralization activities against IL-12 (d) and IL-18 (e) with potencies similar to that of the parental mAbs 1D4.1 (▼) and ABT-325 (▲). 1D4.1-325 DVD-Ig protein also showed tetraivalent dual-specific binding ability as assessed on Biacore by injecting the two antigens sequentially over a DVD-Ig protein-coated chip. (f) When 1D4.1-325 DVD-Ig protein had been saturated by the first antigen (IL-18), the second antigen (IL-12) was injected and a second binding signal was observed. (g) This observation was reproduced when the antigen injection sequence was reversed.

(V_{H1}/V_{L1} on Fig. 1a), and Y is the clone number (or name) of the mAb whose V_H/V_L are internal, to the C termini of the X domains (V_{H2}/V_{L2} on Fig. 1a). The linkers in the light chains and heavy chains of 1D4.1-325 DVD-Ig protein originated from the N-terminal sequences of human C_κ and C_{H1}, respectively. All light chain and heavy chain constant domains are preserved in the 1D4.1-325 DVD-Ig design, as they are critical for the formation of a disulfide-linked full IgG-like molecule. Cotransfection of mammalian cells with expression vectors encoding the 1D4.1-325 DVD-Ig light chain and heavy chain led to the secretion of a single species of an IgG-like molecule with a molecular weight of ~200 kDa (Figs. 1b,c), which separated into a 36-kDa light chain and a 64-kDa heavy chain in reducing condition on SDS-PAGE (Fig. 1c). There were no partial molecules (e.g., free heavy chain, free light chain or heavy chain dimer) detected in the expression medium based on western blot analysis using either anti-Fc (heavy chain) or anti-κ (light chain) antibodies (Fig. 1b). The expression concentrations of 1D4.1-325 DVD-Ig protein from transiently transfected human embryonic kidney 293 cells and COS cells were similar to that of a regular human IgG (within threefold of that of ABT-325), yielding ~5–10 mg/l, which was then easily purified to homogeneity by protein A chromatography. The purified DVD-Ig protein also exhibited physical homogeneity as analyzed by size-exclusion chromatography (Supplementary Fig. 1a online). Subsequent mass spectrometric analysis confirmed the molecular weights of light chain, heavy chain and full-length DVD-Ig protein, as well as a glycosylation profile similar to that of a human IgG (Supplementary Fig. 1b). In addition, all 20 disulfide bonds have been mapped in the DVD-Ig molecule by mass spectrometry, and its charge heterogeneity has been shown to be similar to that of a normal human IgG, as assessed by capillary isoelectric focusing (Supplementary Fig. 1c).

Generation of CHO cell lines

For clinical applications, a majority of therapeutic proteins are produced in stably transfected mammalian cells, with Chinese hamster ovary (CHO) cells as the most commonly used mammalian

host line¹⁰. To address the manufacturing feasibility of DVD-Ig protein, we compared the generation and productivity of 1D4.1-325 DVD-Ig CHO cell lines with that of a conventional human IgG1 antibody, using a commonly adopted methodology assessing stable cell line productivity in a 24-h period^{11,12}. Similar transfection efficiency and initial number of positive clones were identified for both the DVD-Ig and the control mAb (Table 1). Selected clones with a yield >0.8 μg/ml in a 24-h period were then amplified with increasing concentrations of methotrexate (MTX). During this amplification process, a number of adherent 1D4.1-325 DVD-Ig CHO clones was identified, and their expression levels were similar to that of the control human IgG1 (Table 1 and Supplementary Fig. 2 online), with a range of 2–17 μg/ml/24 h, typical for this stage of stable cell line development. Furthermore, the numbers of high-expressing candidate cell lines were comparable between 1D4.1-325 DVD-Ig and the control (Supplementary Figs. 2b,c). These data indicate that there is no observed difference between DVD-Ig and a conventional human IgG with respect to stable cell line development.

Table 1 Comparison of cell line generation between DVD-Ig and a conventional human mAb

	1D4.1-325 DVD-Ig protein	Control mAb ^a
Estimated total clones (100 96-well plates)	6,950	6,504
Positive clones selected	71	78
0 nM MTX	24	28
20 nM MTX	15	17.5
100 nM MTX	12	12
Productivity (μg/ml/day)	2.0–17	1.5–10

Comparable numbers of positive CHO cell clones were obtained at each stage of the CHO stable transfection and amplification process between 1D4.1-325 DVD-Ig protein and a regular human monoclonal antibody. MTX, methotrexate.

^aAverage taken from two transfections. At 0, 20 and 100 nM MTX selection stages, clones with yield > 0.8 μg/ml/day were selected.

Table 2 *In vitro* functional characterization of 1D4.1-325 DVD-Ig protein

Immunoglobulin	Specificity	Affinity ^a			Stoichiometry	Potency ^b (pM)
		k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (pM)		
1D4.1	IL-12	2.60×10^5	2.90×10^{-5}	120	1.8	420
ABT-325	IL-18	3.67×10^5	5.03×10^{-5}	140	1.9	200
1D4.1-325 DVD-Ig protein	IL-12	2.26×10^5	3.01×10^{-5}	130	1.7	220
	IL-18	2.52×10^5	4.01×10^{-5}	160	2.0	120

^aAffinities and binding stoichiometries were measured by Biacore. ^bPotencies were determined by cell-based cytokine neutralization bioassays as described in Methods. Data are representative of three independent experiments.

DVD-Ig protein is dual-specific and tetravalent

The anti-IL-12/IL-18 1D4.1-325 DVD-Ig protein was evaluated for its neutralization potency and binding affinity for both cytokines. DVD-Ig protein inhibited IL-12-induced interferon- γ (IFN- γ) production by phytohemagglutinin (PHA)-blasted human peripheral blood mononuclear cells (PBMC) dose dependently, with an IC_{50} similar to that of the parental antibody 1D4.1 (Fig. 1d and Table 2). 1D4.1-325 DVD-Ig protein also inhibited IL-18-induced IFN- γ production from KG-1 cells, with an IC_{50} similar to that of the parental mAb ABT-325 (Fig. 1e and Table 2). With Biacore affinity testing, 1D4.1-325 DVD-Ig protein exhibited high-affinity for both IL-12 and IL-18, with binding kinetics, stoichiometry and overall affinity constants similar to that of the two parental mAbs (Table 2). Furthermore, the tetravalent binding property of 1D4.1-325 DVD-Ig protein was demonstrated through multiple binding Biacore studies by injecting the two antigens sequentially over a DVD-Ig protein-coated chip. When 1D4.1-325 DVD-Ig protein had been saturated by the first antigen (IL-18), the second antigen (IL-12) was injected and, as expected, a second binding signal was observed (Fig. 1f). By calculating the stoichiometry of the binding events, we determined that 1D4.1-325 DVD-Ig protein bound both antigens simultaneously as a dual-specific tetravalent molecule. This observation was reproduced when the antigen injection sequence was reversed (Fig. 1g).

To assess the general utility of the DVD-Ig technology for development of other mAb pairs, we also produced a different anti-IL-12/IL-18 DVD-Ig protein in which another anti-IL-12 mAb ABT-874 (ref. 13), a fully human mAb with a λ light chain, was paired with ABT-325. The V_L of ABT-874 was fused to the N terminus of the ABT-325 light chain through a 5-amino acid linker (first five residues of human C_L), and the V_H of ABT-874 was fused to the N terminus of ABT-325 heavy chain through a 6-amino-acid linker (first six residues of human $CH1$). This 874-325 DVD-Ig protein (Supplementary Fig. 3a online) was produced in transiently transfected 293 cells (yielding 15 mg/l) and was purified to homogeneity by protein A chromatography (Supplementary Fig. 3b,c). It also retained the binding affinity and neutralization potencies of its two parental antibodies (Supplementary Table 1 online), as well as dual specificity (Supplementary Fig. 2d). The combination of V_L and V_H in the light chain did not alter the activity of the variable domains, indicating that DVD-Ig proteins can accommodate two different types of light chain in its design.

Pharmacokinetic analysis of DVD-Ig protein

The pharmacokinetic properties of the 1D4.1-325 DVD-Ig protein were assessed upon intravenous (i.v.) and subcutaneous (s.c.) dosing (4 mg/kg) in rats. The serum concentrations of DVD-Ig protein were measured by both an IL-12-specific and an IL-18-specific capture enzyme-linked immunosorbent assay (ELISA), with anti-human Fc detection. 1D4.1-325 DVD-Ig protein serum concentrations were very

similar when determined by the two different ELISA methods, indicating that the molecule was intact and capable of binding both antigens in the presence of serum. Upon intravenous dosing, 1D4.1-325 DVD-Ig protein exhibited a biphasic pharmacokinetic profile, consisting of a distribution phase and followed by an elimination phase (Fig. 2), similar to the pharmacokinetic profile of conventional IgG molecules. The pharmacokinetic parameters calculated based on the two different analytical methods were very similar (Table 3). As demonstrated by these results, the *in vivo* properties of 1D4.1-325 DVD-Ig protein are very similar to a conventional IgG molecule. Moreover, the main pharmacokinetic parameters of 1D4.1-325 DVD-Ig protein in rat were very close to those of the parental mAbs ($T_{1/2}$, 13.6 d for 1D4.1 and 15.3 d for ABT-325), indicating a potential for therapeutic applications using comparable dosing regimens.

DVD-Ig protein blocks cytokine action *in vivo*

We first compared the *in vivo* biologic activity of 1D4.1-325 DVD-Ig protein in a human PBMC-engrafted severe combined immunodeficient (SCID) mouse model with the combination of anti-IL-12 and anti-IL-18 mAbs. Synergistic effects of IL-12 and IL-18 in inducing IFN- γ production have been observed in several biologic systems^{14–16}, and targeting both cytokines may be beneficial for autoimmune indications¹⁷. In the human PBMC-grafted SCID mouse model¹⁸, 1D4.1 and ABT-325 at a 250- μ g/mouse dose inhibited

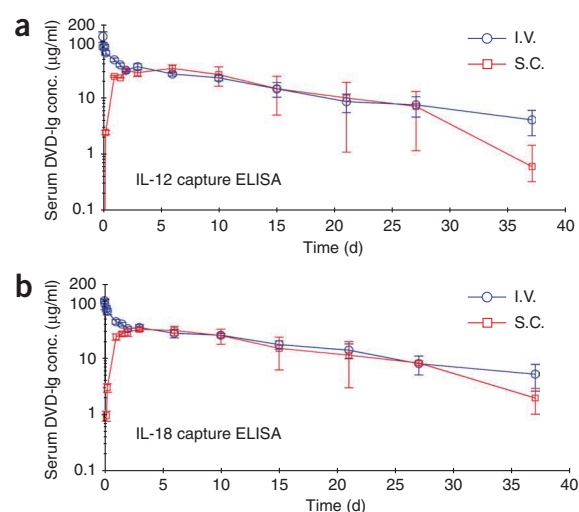


Figure 2 Pharmacokinetic profile of 1D4.1-325 DVD-Ig protein in rats. Serum concentrations ($\pm$ s.d.) time profile of anti-IL-12/IL-18 1D4.1-325 DVD-Ig protein after a 4-mg/kg intravenous (blue circles, $n = 4$) or subcutaneous (red squares, $n = 2$) dose in male Sprague-Dawley rats. Detection was carried out using IL-12 capture (a) or IL-18 capture (b) ELISA.

Table 3 Pharmacokinetic parameters of 1D4.1-325 DVD-Ig protein in rats

Route	Parameter ^a	1D4.1-325 DVD-Ig protein	
		IL-12 capture	IL-18 capture
i.v.	CL (ml/h/kg)	0.26 ± 0.01	0.23 ± 0.02
	T _{1/2} (d)	11.2 ± 1.33	11.8 ± 1.79
	V _{ss} (ml/kg)	90.4 ± 9.74	88.8 ± 6.48
	Vz (ml/kg)	97.1 ± 10.09	89.2 ± 7.26
	AUC (day-mg/ml)	665 ± 20	753 ± 65.9
	MRT (h)	15.2 ± 1.84	16.9 ± 2.38
s.c.	T _{max} (d)	6	4.5
	C _{max} (μg/ml)	33.4	32.3
	T _{1/2} (d)	11.3	10.9
	AUC (day-mg/ml)	612	640
	F (%)	92	85

^aNumbers are the average of four animals for i.v. (with ± s.e.m.) administration and average of two animals for s.c. administration. AUC = area under the plasma-concentration-versus-time curve; F = bioavailability; MRT = mean residence time; Vz = volume of distribution.

Staphylococcus aureus dried cell (SAC)-induced IFN- γ production by 78% and 92%, respectively, which was the maximum inhibition achievable with each individual mAb alone (Fig. 3). A combination of 1D4.1 and ABT-325 at a 250 μ g/mouse dose, however, almost completely abrogated SAC-induced IFN- γ production (98%), demonstrating the benefit of combination treatment. The 1D4.1-325 DVD-Ig protein at a 250- μ g/mouse dose achieved the same inhibitory effect as the combination of 1D4.1 and ABT-325. Similarly, the *in vivo* activity of the second anti-IL-12/IL-18 874-325 DVD-Ig protein was also demonstrated in the human (donor no. 3) PBMC-grafted SCID mouse model (Supplementary Fig. 4 online). ABT-874 and ABT-325 at a 250- μ g/mouse dose can inhibit SAC-induced IFN- γ production by 81% and 75%, respectively, which was the maximum inhibition achievable with each individual mAb alone. A combination of ABT-874 and ABT-325 at a 250- μ g/mouse dose each, however, almost completely abrogated SAC-induced IFN- γ production (98%). The 874-325 DVD-Ig protein at a 250- μ g/mouse dose (all dosing routes including i.v., s.c. and intraperitoneal (i.p.)) achieved the same inhibitory effect as the combination of ABT-874 and ABT-325.

Therapeutic efficacy in preclinical disease model

We next assessed therapeutic efficacy of the DVD-Ig protein in a preclinical animal disease model. Previous studies have shown that the blockade of both IL-1 α and IL-1 β with a combination of anti-mouse

Figure 4 Anti-mIL-1 α / β 10G11-9H10 DVD-Ig protein shows comparable efficacy to monospecific anti-IL-1 α and anti-IL-1 β antibody combinations in collagen-induced arthritis. (a) Mouse anti-mIL-1 α / β 10G11-9H10 DVD-Ig protein and the monospecific antibody combinations show comparable efficacy as assessed by mean arthritic score (**P* < 0.05 by nonparametric Mann-Whitney test) or (b) paw-swelling (**P* < 0.05 by student *t*-test). Histology corroborates the clinical data. (c–f) Representative hematoxylin and eosin stain of various treatment groups. (c) PBS group, showing bone erosion (*), cartilage destruction (arrows heads), and synovial hyperplasia with inflammation (I), whereas (d) the parent monospecific anti-IL-1 α and anti-IL-1 β antibody combination, (e) the monospecific anti-IL-1 α and anti-IL-1 β antibody combination with similar potencies to the 10G11-9H10 DVD-Ig protein, and (f) 10G11-9H10 DVD-Ig protein treatment groups were protected from bone and cartilage damage and had near normal synovium. Scale bar, 200 μ m.

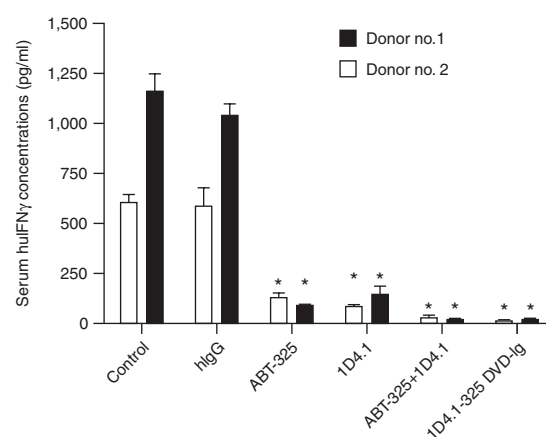
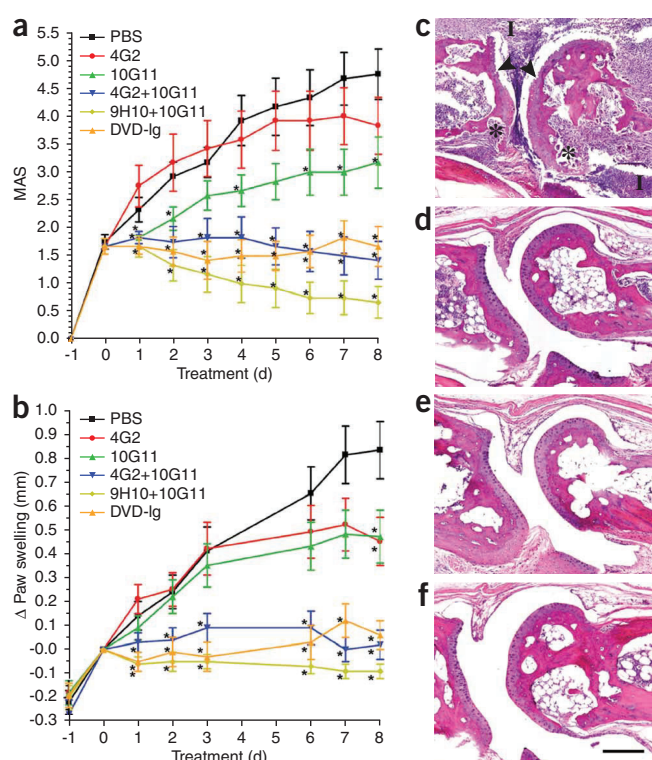


Figure 3 *In vivo* activity of 1D4.1-325 DVD-Ig protein in a human PBMC-engrafted SCID mouse model. Anti-IL-18 mAb ABT-325, anti-IL-12 mAb 1D4.1, combination of ABT-325 and 1D4.1, 1D4.1-325 DVD-Ig protein, PBS (control), and human IgG (hlgG) were administered intraperitoneally (*n* ~4–7 mice per group), followed by human PBMC (two different donors) engraftment and *Staphylococcus aureus* dried cells challenging in SCID mice as described in Methods. All animals were terminally bled and serum IFN- γ concentrations (mean ± s.d.) were measured. **P* < 0.002 compared to control and hlgG group.

IL-1 α and IL-1 β antibodies was significantly more efficacious than a single antibody alone in the mouse collagen-induced arthritis (CIA) model for rheumatoid arthritis¹⁹. We engineered a mouse anti-mouse IL-1 α / β DVD-Ig protein (Supplementary Fig. 5a online) that could neutralize both mouse IL-1 α and IL-1 β using a mouse anti-mouse IL-1 α mAb (9H10) and a mouse anti-mouse IL-1 β mAb (10G11), which exhibited antigen neutralization potencies of 0.2 nM (mIL-1 α) and 0.4 nM (mIL-1 β), respectively. The resulting mouse anti-mIL-1 α / β



10G11-9H10 DVD-Ig protein was produced from mammalian cells, yielding ~15 mg/l (Supplementary Fig. 5). It was characterized by Biacore and MRC-5 bioassay²⁰, with neutralizing potencies of 2.0 nM for mIL-1 α and 0.8 nM for mIL-1 β (Supplementary Table 2 online). As controls in the CIA study, the combination of the parental mAbs (9H10 and 10G11) was included. Considering the tenfold difference in anti-mIL-1 α potency between 9H10 and the 10G11-9H10 DVD-Ig protein, we also included a different anti-IL-1 α mAb, 4G2, with a potency (2.5 nM) similar to that of the 10G11-9H10 DVD-Ig protein. Our results demonstrate that therapeutic administration of 10G11-9H10 DVD-Ig protein significantly inhibited the progression of established arthritis ($P < 0.05$ versus vehicle) as assessed by the mean arthritic score (Fig. 4a) and paw swelling (Fig. 4b), with an efficacy comparable to the combination of the parental mAbs 9H10 and 10G11, as well as potency-matching mAb combination 4G2 and 10G11. Monospecific mAbs alone did not show any significant efficacy. There was no statistically significant difference between the two different mAb combination groups. These results were further corroborated by histologic findings demonstrating that the anti-mIL-1 α/β 10G11-9H10 DVD-Ig protein was as efficacious as the monospecific mAb combinations in protecting mice from bone and cartilage damage after the onset of disease (Fig. 4c-f). A lower dosage of the 10G11-9H10 DVD-Ig protein treatment was not efficacious in this study (data not shown).

DISCUSSION

We describe here a generally applicable approach to develop tetra-valent, dual-specific, Ig-like molecules with considerable therapeutic potential. Biacore analysis of multiple binding stoichiometry revealed that 1D4.1-325 DVD-Ig protein was capable of binding two IL-12 and two IL-18 molecules, indicating that each binding domain could function independently without posing substantial steric hindrance to one another. This is not obvious given the antigen-binding nature of an IgG and the notion that any large structure close to a complementarity-determining region may disrupt its interaction with the antigen. The structural flexibility of IgG, which is of functional significance for antigen binding, has been described²¹. With proper peptide linkages between the two variable domains in both heavy chain and light chain, the various motions within the Fab region (Fab elbow bend, and Fab arm waving and rotation) may provide sufficient structural freedom in a DVD-Ig protein, enabling its dual-binding capability. Based on our working experience of constructing DVD-Ig molecules using several different pairs of mAbs, we believed it was important to optimize the orientation of the two variable domains, to ensure that each V_H/V_L domain would best preserve the original antigen-binding activity. In most cases, the variable domains that bind to an antigen of larger molecular size have been constructed on top (N terminus) of the DVD-Ig molecule to avoid structural constraints. For example, the anti-IL-12/IL-18 DVD-Ig molecules (for both 1D4.1-325 and 874-325 DVD-Ig proteins) preserve the affinities of both parental mAbs well in the current V₁₂-V₁₈-constant orientation; however, a two- to fivefold loss of affinity was observed for anti-IL-12 in the V₁₈-V₁₂-constant orientation. In case of anti-mIL-1 α/β 10G11-9H10 DVD-Ig protein, a tenfold decrease of potency was observed for anti-mIL-1 α even after construct optimization, indicating that certain sequence-derived properties of the parental mAb (that is, 9H10) can affect DVD-Ig protein function. As each DVD-Ig protein is unique and its properties are often related to (or limited by) the properties of the parental mAbs, including affinity, potency, physicochemical and pharmacokinetic characteristics, it will be beneficial in practice to have several mAbs with high

affinity and of distinct lineages as building blocks for DVD-Ig protein construct optimization.

The linkers between the two variable domains are critical to both functional activity and efficient expression of DVD-Ig proteins. We chose the first five and six amino acids from the N termini of human C _{κ} and C_{H1} domains, respectively, as the linker sequences for most of our constructs. Extensive Fab crystal structure studies in the literature have well documented that these regions adopt a flexible, loop-like orientation without any strong secondary structure. This makes them suitable for functioning as linkers between structural domains. In addition, they are natural sequence extensions of the variable domains within the IgG molecule, potentially eliminating possible instability and immunogenicity issues that may otherwise be caused by using non-Ig-derived linker sequences. Although immunogenicity cannot be addressed adequately in preclinical animal models, we have attempted to delineate the *in vivo* structural and functional integrity of DVD-Ig protein, with a focus on the linker regions. The IL-12- and IL-18-capturing ELISAs described identical pharmacokinetic profiles of 1D4.1-325 DVD-Ig protein throughout the course of the 37-d study, indicating that the top variable domains had not been cleaved off from the 1D4.1-325 DVD-Ig molecule and that the linkers remained intact and stable *in vivo*. We have also used linkers of up to 13 amino acids successfully. In many cases, longer linkers may result in better conservation of parental domain activities, particularly for the lower domain. However, extra-long linkers should be avoided, as they may be prone to proteolysis. A balance between functional activity and *in vitro/in vivo* stability needs to be considered in selecting the linker size for any DVD-Ig protein construct.

In the design of DVD-Ig protein, we were interested in maintaining the complete human Fc region for potentially desired effector functions—antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity—for various indications. Many mAb-derived bispecific molecules with intact human Fc have been described^{6,7}. The pharmacokinetic property of DVD-Ig protein has demonstrated a critical improvement over previously described multi-specific antibody-based molecules. For example, a bispecific, fully human di-antibody molecule appeared to lose its activity 6 h after injection, owing to *in vivo* dissociation of the light chain and its rapid clearance, resulting in T_{1/2} < 24 h²². A recently reported single-domain-IgG fusion protein, which required a single-domain binding unit, was not stable *in vitro* in 10% serum, and no subsequent *in vivo* pharmacokinetics or efficacy were demonstrated²³. Most of these bispecific molecules were not adequately evaluated for their *in vivo* properties, often because of either production/purification issues or poor *in vitro* stability²⁴. The rat pharmacokinetic system is commonly used in the pharmaceutical industry for preclinical evaluation of therapeutic mAbs, and it correlates well with the pharmacokinetic profile of mAbs in humans. The relatively long half-life and low clearance of DVD-Ig protein will drive its therapeutic utility for chronic indications, potentially with a dosing regimen similar to that for a therapeutic mAb. In addition, DVD-Ig protein, which is 50 kDa larger than an IgG, seemed to penetrate into the tissues comparably to an IgG, based on its mAb-like volume-of-distribution parameter from the pharmacokinetic study. Furthermore, the therapeutic efficacy of the mouse anti-mIL-1 α/β DVD-Ig protein in the CIA study also suggested its presence in the joints, as drug penetration into the site of action (synovial cavity) is critical for achieving efficacy in various experimental animal models of inflammatory arthritis²⁵.

Another critical feature of DVD-Ig protein is the ease of production and purification. The issue of manufacturing has been a major hurdle for many bispecific or multi-specific antibody-derived molecules^{7,26}.

In contrast, the DVD-Ig proteins described here have expression levels comparable to those of conventional IgG molecules in mammalian cells, in both transient and stable systems. Furthermore, characterization of the DVD-Ig protein has shown intact disulfide bonds in all 20 locations and the functional integrity of its four antigen-binding domains has demonstrated a correct structural folding of each domain. In addition, the DVD-Ig protein accurately matches the functional V_H/V_L pairs and is devoid of domain mismatches or smaller fragments of partial molecules, which have been described in single-chain or diabody-derived constructs⁷. The homogeneity of DVD-Ig protein expression in mammalian systems should enable its production scale-up and purification under existing antibody manufacturing processes commonly adopted across the biopharmaceutical industry.

DVD-Ig technology appears to have the versatility to combine mAbs of different origin (human, humanized, chimeric or murine) and isotype (κ and λ), as well as mAbs that target proteins of different sizes (molecular weight from 17 kDa for IL-1 to 70 kDa for IL-12 have been demonstrated). In addition, DVD-Ig protein can target two soluble proteins (that is, IL-12 and IL-18) as well as two cell-surface molecules. We have constructed an anti-CD20/CD3 DVD-Ig protein using an anti-CD20 mAb (1F5)²⁷ and an anti-CD3 mAb (OKT3)²⁸, and preliminary analysis of 1F5-OKT3 DVD-Ig protein indicated that it was active in binding both CD20 and CD3 on human B and T cells, respectively (Supplementary Fig. 6 online). As we are still in the early stage of investigating DVD-Ig proteins against membrane proteins, studies to address their binding affinities and functional abilities in binding CD20 and CD3 simultaneously to induce redirected cytotoxicity are currently in progress. Nevertheless, this molecule provides proof of concept for potentially targeting two cell-surface receptors with a DVD-Ig protein.

Antibody-derived molecules with multiple specificities have been an active area of research for the past two decades. Extensive efforts have focused on the generation of various forms and/or fragments of bispecific antibodies for cancer therapy through the mechanism of redirected cytotoxicity^{29–31}. In recent years, it has also become increasingly apparent that blocking multiple-disease mediators will be critical and more efficacious than inhibiting only one target in a complex disease state²⁴. Multi-specific biologics present an intriguing prospect for developing such next-generation therapies. They offer functionalities and targeting mechanisms unattainable by a combination of two antibodies, including increased functional affinity through avidity³², receptor activation³³, delivery of toxins³⁴, tissue-specific targeting³⁵ and immunodiagnostics³⁶. For drug development, these multi-specific biologics can also be used as improved, second-generation biologics by adding a new component onto an already marketed therapeutic antibody. The DVD-Ig protein technology offers an approach for combining two existing antibodies into one dual-specific molecule. Our results have demonstrated the drug-like properties and feasibility of early-stage manufacturing of the DVD-Ig protein.

METHODS

Construction, expression and purification. The DVD-Ig protein was designed as an IgG-like molecule, except that each light chain and heavy chain of a DVD-Ig protein molecule contains two variable domains in tandem (Fig. 1a), instead of one variable domain as in an IgG. To demonstrate and validate this technology, we engineered from two high-affinity human mAbs, 1D4.1 and ABT-325, an anti-IL-12/IL-18 1D4.1-325 DVD-Ig protein. With overlapping polymerase chain reaction, the V_H and V_L of 1D4.1 were fused to the N terminus of the heavy chain and light chain of ABT-325, respectively, through a short linker (Fig. 1a). The linkers between the two variable domains in both

the heavy chain (the linker sequence is ASTKGP) and the light chain (the linker sequence is TVAAP) were selected from the N termini of human C_{H1} and C_{κ} sequences, respectively. The heavy chain and the light chain of the 1D4.1-325 DVD-Ig molecule were subcloned into mammalian expression vectors (Invitrogen) and were transiently co-expressed in COS cells or human embryonic kidney 293 cells (ATCC), followed by protein A purification (Pierce Chemicals) according to manufacturer's instructions. The expression media were analyzed by western blot using a horseradish peroxidase (HRP)-conjugated anti-human Fc antibody (Pierce Chemicals) and an HRP-conjugated anti-human κ chain antibody (Dako). Furthermore, expression concentrations of DVD-Ig protein and human mAbs were determined by human Ig ELISA (R&D Systems). The purified 1D4.1-325 DVD-Ig protein was analyzed by SDS-PAGE and quantified by A280 and bicinchoninic acid (BCA) (Pierce). The cloning and production of 874-325 DVD-Ig protein were completed similarly, with the exception of using ABT-874 instead of 1D4.1. Likewise, the mouse anti-mIL-1 α / β 10G11-9H10 DVD-Ig protein was constructed with a mouse anti-mouse IL-1 α mAb (9H10) and a mouse anti-mouse IL-1 β mAb (10G11), which were generated through hybridoma technology by immunizing IL-1 α / β double knockout (KO) mice with mouse recombinant IL-1 α and IL-1 β , respectively. In an *in vitro* bioassay in which MRC-5 cells produce IL-8 in response to IL-1 (ref. 20), the anti-mIL-1 α and the anti-IL-1 β mAbs had neutralizing potencies of 0.2 nM and 0.4 nM, respectively. In this fully murine 10G11-9H10 DVD-Ig construct (with mouse Fc), the heavy chain and light chain variable domains of the two parent mAbs were combined in tandem by a linker (with a domain orientation of $V\beta$ -linker- $V\alpha$ -constant region for both heavy chain and light chain) and then were coexpressed in a mammalian cell line to generate the mouse 10G11-9H10 DVD-Ig protein. The fully mouse IL-1 α / β 10G11-9H10 DVD-Ig protein was characterized by MRC-5 bioassay to be dually functional in neutralizing both mIL-1 α (potency [IC₅₀] = 2.0 nM) and mIL-1 β ([IC₅₀] = 0.8 nM). The anti-CD20/CD3 1F5-OKT3 DVD-Ig protein was constructed using an anti-CD20 mAb (1F5)²⁷ and an anti-CD3 mAb (OKT3)²⁸, and the heavy chain and light chain variable domains of the two parent mAbs were combined in tandem by a linker (with a domain orientation of V_{1F5} -linker- V_{OKT3} -human constant region for both heavy chain and light chain) and then were coexpressed in a mammalian cell line to generate the chimeric 1F5-OKT3 DVD-Ig protein (yielding 3.5 μ g/ml). Preliminary functional analysis by FACS was performed using human Jurkat (T) and Raji (B) cells, with a secondary fluorescein isothiocyanate conjugated-labeled goat anti-mouse IgG for detection. Both the protein A purified 10G11-9H10 DVD-Ig protein and the 1F5-OKT3 DVD-Ig protein exhibited minor aggregation (4–5%) as assessed by size-exclusion chromatography. This degree of aggregation is common to regular monoclonal antibody preparations that are purified by research grade protein A purification process with PBS buffer.

Generation of CHO cell lines. Cell lines stably expressing 1D4.1-325 DVD-Ig were generated as previously described³⁷. Briefly, dihydrofolate reductase (DHFR)-deficient CHO dux-B11 cells were plated at a density of 1.25×10^6 cells/10 cm dish, with alpha medium containing 10% FBS (Invitrogen) 24 h before transfection. Cells from each 10-cm dish were transfected with 25 μ g of 1D4.1-325 DVD-Ig construct DNA in a $CaCl_2$ and $2 \times$ HEPES-containing solution. After 24 h the cells were split into 96-well plates at a density of 200 cells/well and grown in alpha medium containing 5% FBS for a period of 2 weeks, wherein transfectants were assessed by human Ig ELISA after a 24-h incubation period with fresh medium to determine expression concentrations of DVD-Ig. During the amplification process, selected transfectants were grown in MTX. Initially, 20 nM of MTX was added to the growth medium and clones were allowed to adapt for three cell passages. Upon routine assessment by ELISA, clones that showed increases in expression levels were further amplified by addition of 100 nM of MTX (two to three cell passages).

Binding affinity measurements by Biacore. The kinetics of 1D4.1-325 DVD-Ig protein binding to recombinant human IL-12 and IL-18 was determined by surface plasmon resonance-based measurements with a Biacore 3000 instrument (Biacore AB) using standard procedures similar to mAb analysis. Briefly, goat anti-human IgG Fc γ polyclonal antibody (Pierce Chemicals) was directly immobilized across a biosensor chip, and 1D4.1-325 DVD-Ig protein or mAb samples were injected over reaction matrices at a flow rate of 5 μ l/min. The

association and dissociation rate constants, k_{on} ($\text{M}^{-1}\text{s}^{-1}$) and k_{off} (s^{-1}), respectively, were determined with a continuous flow rate of 25 $\mu\text{l}/\text{min}$. Rate constants were derived by making kinetic binding measurements at ten different antigen concentrations, ranging from 1.25 to 1,000 nM. The equilibrium dissociation constant K_D (M) of the reaction between 1D4.1-325 DVD-Ig protein and IL-12/IL-18 was then calculated from the kinetic rate constants by the following formula: $K_D = k_{\text{off}}/k_{\text{on}}$. The apparent stoichiometry of the captured 1D4.1-325 DVD-Ig protein-antigen complex was calculated under saturating binding conditions (steady-state equilibrium).

Cell-based functional assays. IL-12 bioassay. The anti-IL-12 neutralization activity of 1D4.1-325 and 874-325 DVD-Ig proteins were analyzed by PHA blast human PBMCs, which have been shown to produce IFN- γ in response to IL-12 stimulation³⁸. DVD-Ig protein at a final concentration range of 10^{-7} M– 10^{-14} M were preincubated for 1 h at 37 °C with 50 μl of 800-pg/ml human IL-12 in RPMI complete medium in a 96-well, flat-bottom microtiter plate. PHA blast cells (2.5×10^6 cells/ml, 100 $\mu\text{l}/\text{well}$) were added to the DVD-Ig protein + IL-12 mixture, followed by incubation for 18 h at 37 °C. The final concentration of human IL-12 in the assay was 200 pg/ml. The IFN- γ concentrations in the supernatants were measured by human IFN- γ ELISA. 1D4.1 and human IgG were also analyzed in parallel as positive and negative controls.

IL-18 bioassay. The anti-IL-18 neutralization activity of 1D4.1-325 and 874-325 DVD-Ig protein was analyzed by a human leukemic cell line KG-1 (ATCC). IL-18 drives IFN- γ production from KG-1 cells in the presence of TNF α ³⁹. Briefly, IL-18 (in a final concentration of 2 ng/ml) was preincubated with DVD-Ig protein (in final concentrations between 0 and 10 $\mu\text{g}/\text{ml}$) at 37 °C for 1 h and then were added to KG-1 cells ($3 \times 10^6/\text{ml}$) in RPMI medium containing 10 ng/ml of TNF, followed by incubation at 37 °C for 16 to 20 h. The culture supernatants were collected, and human IFN- γ production in each sample was determined by ELISA. ABT-325 and human IgG were also analyzed in parallel as positive and negative controls.

IL-1 bioassay. The potency of anti-IL-1 α/β 10G11-9H10 DVD-Ig protein was measured by MRC-5 bioassay. The MRC-5 cell line (ATCC) is a human lung fibroblast cell line that produces IL-8 in response to human IL-1 α and IL-1 β in a dose-dependent manner²⁰. 10G11-9H10 DVD-Ig protein (10^{-7} – 10^{-12} M in 50 μl) in minimal essential medium (MEM) 10% FBS was added to a 96-well plate and preincubated with mIL-1 α or mIL-1 β (200 pg/ml in 50 μl) for 1 h at 37 °C. MRC-5 cells at a concentration of $10^5/\text{ml}$ were then added (100 μl) to all wells, and the plates were incubated overnight at 37 °C in a 5% CO_2 incubator. The supernatants were harvested, and human IL-8 production was measured by standard ELISA. DVD-Ig protein IC_{50} was determined by its ability to inhibit cytokine production in these bioassay systems using Prism software (GraphPad Software).

DVD-Ig protein pharmacokinetics in rats. Pharmacokinetic properties of 1D4.1-325 DVD-Ig protein were assessed in male Sprague-Dawley rats. The animals were cared for according to the National Institutes of Health guidelines; they were housed in rooms maintained at constant temperature and humidity under a 12-h light/dark cycle, were fed with normal rodent chow, and were allowed food and water *ad libitum*. An Institutional Animal Care and Use Committee (IACUC) approved all animal handling and experimental protocols. 1D4.1-325 DVD-Ig protein and the mAbs were administered to male Sprague-Dawley (SD) rats at a single intravenous dose of 4 mg/kg via a jugular cannula or subcutaneously under the dorsal skin. Serum samples were collected at different time points over a period of 37 d and were analyzed by human IL-12 capture and/or human IL-18 capture ELISAs. Briefly, ELISA plates were coated with goat anti-biotin antibody (5 $\mu\text{g}/\text{ml}$, 4 °C, overnight), blocked with Superblock (Pierce Chemicals), and incubated with biotinylated human IL-12 (IL-12 capture ELISA) or IL-18 (IL-18 capture ELISA) at 50 ng/ml in 10% Superblock Tris-Tween buffered saline (TTBS) at 25 °C for 2 h. Serum samples were serially diluted (0.5% serum, 10% Superblock TTBS) and incubated on the plate for 30 min at 25 °C. Detection was carried out with HRP-labeled goat anti-human antibody, and concentrations were determined with the help of standard curves using the 4-parameter logistic fit. Several animals, especially in the subcutaneous group, exhibited a sudden drop in mAbs/1D4.1-325 DVD-Ig protein concentrations after day 10, probably because they developed an

anti-human response. These animals were eliminated from the final calculations. Values for the pharmacokinetic parameters were determined by a noncompartmental model using WinNonlin software (Pharsight Corporation).

Human PBMC-engrafted mouse SCID model. Female CB.17 SCID mice 6 to 8 weeks old (Taconic Labs) were used for human PBMC-engrafted SCID mouse models. Abbott Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited and Abbott Institutional Animal Care and Use Committee (IACUC) approved all of the procedures. CB.17 SCID mice (6–7 for each groups) were administered with 1D4.1, ABT-325, 1D4.1-325 DVD-Ig protein, 1D4.1 and ABT-325 in combination, PBS, or control human IgG in a volume of 300 μl i.p. 24 h before engraftment of 3×10^7 human PBMCs in a volume of 300 μl i.p. Fifteen minutes after human PBMC engraftment, 1 mg/kg of SAC (Sigma) suspended in 0.9% isotonic saline was injected in a volume of 300 μl i.p. to stimulate engrafted human PBMCs to produce human IFN- γ . Eighteen hours after SAC stimulation, blood was collected by cardiac puncture and serum was prepared to determine human IFN- γ concentrations using a human IFN- γ ELISA kit. The percentages of inhibition of induced IFN- γ were calculated, and the statistical significance of each group was analyzed by two-tailed test. Similar studies were also conducted with 874-325 DVD-Ig protein, including groups of mice pretreated with ABT-874, ABT-325, 874-325 DVD-Ig protein, ABT-874 and ABT-325 in combination, PBS, or control human IgG in a volume of 300 μl i.p. or 100 μl i.v. 1 h before or in a volume of 100 μl s.c. 24 h before i.p. engraftment of 3×10^7 human PBMCs.

Collagen-induced arthritis. The Abbott Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited and the IACUC approved all procedures. The protocol for the induction of collagen-induced arthritis was described previously⁴⁰. Male DBA/J mice (8- to 10-weeks old, Jackson Laboratories) were immunized intradermally at the base of the tail with 100 μl of emulsion containing 100 μg of type II porcine collagen dissolved in 0.1 N of acetic acid and 100 μg of *Mycobacterium tuberculosis* H37Ra (Complete Freund's Adjuvant, Difco). Twenty-one days after immunization with collagen, mice were boosted with 1 mg i.p. of zymosan (Sigma). After the boost, mice were monitored daily for arthritis. Each paw was scored by the following criteria: 0 = normal; 1 = swelling in one site, foot or ankle; 2 = swelling in foot and ankle; and 3 = ankylosis. Scores were summed for each animal, and the total average of all animals in each group was expressed as the mean arthritic score. In addition to clinical scores, mice were also evaluated for paw-edema using caliper. Mice were enrolled for the study between days 24 and 28 at the first clinical signs of disease. At the termination of the experiment, four paws from various groups were harvested and stored in 10% neutral buffered formalin for histopathology.

Histopathology. Formalin-fixed paws were sectioned and stained with Gills 3 hematoxylin (Richard-Allan Scientific) and eosin with phloxine (Newcomer Supply). Severity of disease was evaluated histologically using the following criteria: 0 = normal; 1 = minimal change; 2 = mild change; 3 = moderate change; and 4 = severe change. Scores were summed for each animal, and the total was expressed as an average of all animals in each group.

Treatment in collagen-induced arthritis. Mice with first clinical signs of disease were dosed i.p. two times a week with either the parent monospecific anti-IL-1 α (clone 9H10) at 225 $\mu\text{g}/\text{mouse}$ or another monospecific anti-IL-1 α (clone 4G2) at 500 $\mu\text{g}/\text{mouse}$ and anti-IL-1 β (clone 10G11) at 112.5 $\mu\text{g}/\text{mouse}$ in combination. The doses for the combinations of the monospecific anti-IL-1 α and anti-IL-1 β antibodies in CIA were determined previously. The anti-IL-1 α/β 10G11-9H10 DVD-Ig protein was dosed i.p. three times a week at 156.25 $\mu\text{g}/\text{mouse}$ and 625.0 $\mu\text{g}/\text{mouse}$. Negative control included mice treated with PBS alone.

Statistical analysis. Summary statistics are reported as the mean and the standard error for each treatment group in the CIA studies. For mean arthritic scores, the non-parametric Mann-Whitney test was used to identify significant differences between experimental groups compared with the arthritis, vehicle-treated control group. Statistical significance is achieved if $P < 0.05$. Statistics were generated using Statistica software (StatSoft). Paw-swelling summary statistics are also reported as mean and standard error. A Student t -test was

used to identify significant ($P < 0.05$) differences between the groups. Statistics were generated using Microsoft Excel software (Microsoft Corporation).

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>.

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