CODV-Ig, a universal bispecific tetravalent and multifunctional immunoglobulin format for medical applications

Anke Steinmetz, François Vallée, Christian Beil, Christian Lange, Nicolas Baurin, Jochen Benning, Cécile Capdevila, Carsten Convey, Alain Dupuy, Paul Ferrari, Alexey Rak, Peter Wonerow, Jochen Kruip, Vincent Mikolc, and Ercole Rao

ABSTRACT
Bispecific immunoglobulins (Igs) typically contain at least two distinct variable domains (Fv) that bind to two different target proteins. They are conceived to facilitate clinical development of biotherapeutic agents for diseases where improved clinical outcome is obtained or expected by combination therapy compared to treatment by single agents. Almost all existing formats are linear in their concept and differ widely in drug-like and manufacture-related properties. To overcome their major limitations, we designed cross-over dual variable Ig-like proteins (CODV-Ig). Their design is akin to the design of circularly closed repeat architectures. Indeed, initial results showed that the traditional approach of utilizing (G4S), linkers for biotherapeutics design does not identify functional CODV-Igs. Therefore, we applied an unprecedented molecular modeling strategy for linker design that consistently results in CODV-Igs with excellent biochemical and biophysical properties. CODV architecture results in a circular self-contained structure functioning as a self-supporting truss that maintains the parental antibody affinities for both antigens without positional effects. The format is universally suitable for therapeutic applications targeting both circulating and membrane-localized proteins. Due to the full functionality of the Fc domains, serum half-life extension as well as antibody- or complement-dependent cytotoxicity may support biological efficiency of CODV-Igs. We show that judicious choice in combination of epitopes and paratope orientations of bispecific biotherapeutics is anticipated to be critical for clinical outcome. Uniting the major advantages of alternative bispecific biotherapeutics, CODV-Igs are applicable in a wide range of disease areas for fast-track multi-parametric drug optimization.

Introduction
Bispecific antibodies may target soluble ligands or membrane receptors to modulate signaling, or they may bind cell surface proteins, such as in effector cell retargeting strategies. Two bispecific antibody therapeutics, catumaxomab (Removab®) and blinatumomab (Blincyto®), have been approved, and over ten bispecific antibodies are currently in clinical development for treatment of inflammatory diseases, hemophilia A, or cancers.

The drug-like properties of bispecific biotherapeutics described in the literature vary widely. Molecular weights range from ~55 kDa to over 300 kDa, their valences for antigen binding are from two to six, and serum half-lives are reported to be between ~0.5 hours and ~2 weeks. Each format presents a particular strength in physicochemical properties, manufacturing, formulation, pharmacokinetics, or pharmacodynamics while being limited in the other evaluation criteria. Sustained structural and functional homogeneity throughout manufacture, storage, and in vivo exposure is critical to their successful therapeutic application, but recognized in many bispecific formats as limiting factor. For example, any format isolating a variable fragment (Fv) by replacing its adjacent constant fragment (Fc) with a peptide linking heavy or light chain Fv domains is associated with decreased Fv stability. Introduction of disulfide bridges or improved interface interactions stabilize Fv dimerization in specific cases; however, such strategies remain associated with loss of antigen affinity or increased aggregation propensity. Asymmetric bispecific antibodies including Fc domains give rise to improperly paired side-products even when optimized. Thus, they generally require further engineering, extensive purification, or special production systems imposing specific limitations. Furthermore, their avidity per antigen is reduced compared to the parental antibodies.

Symmetric Fc containing bispecific immunoglobulins (Igs) overcome limitations of asymmetric constructs. Improperly paired side-products are avoided and avidity per antigen is preserved at the parental level. The dual-variable-domain IgG...
(DVD-Ig) format overcomes limitations of Fv instability by N-terminally extending both heavy and light chains of a natural antibody with another Fv fragment (Fig. 2). However, some DVD-Igs show a positional effect; thus, they maintain antigen-afﬁnity at the level of the parental antibody only at the outer, N-terminal Fv (Fv1). Antigen afﬁnity at the inner Fv (Fv2) is signiﬁcantly reduced. Structural studies suggest that Fv1 may hinder antigen access by swinging “like a bucket” over the antigen-binding region of Fv2.

Biotherapeutic agents require a proper balance of target tissue penetration and maintenance of therapeutic concentrations at the site of action. Low molecular weight bispesific IgGs devoid of Fc domains aim at superior pharmacodynamics by increased target tissue penetration. They potentially require continuous intravenous infusion over weeks to maintain therapeutic serum concentrations. The high molecular weight of bispesific agents including Fc domains may limit tissue penetration. Nevertheless, such constructs preserve the potential to bind to Ig receptors (FcRs). As a consequence, they may have extended serum half-lifes by endocytic salvage via neonatal FcR (FcRn), which may compensate for slow tissue penetration. Furthermore, they may elicit antibody-dependent cell-mediated cytotoxicity or phagocytosis (ADCC or ADCP, respectively) via Fcγ receptors (FcγRs), which is important for cancer biotherapeutics. Fully functional Fc domains may also confer the potential to trigger the classical pathway of complement-dependent humoral response, and thereby elicit complement-dependent cytotoxicity (CDC). To overcome major limitations of existing bispesific biotherapeutics and to create a universally applicable format that can be ﬁne-tuned in multi-parametric drug optimization, we designed the CODV format to serve as a versatile platform for the development of bispesific agents.

Results

Inspired by the structures of natural antibodies, different types of CODV biotherapeutics may be engineered. Both heavy and light chains of a ﬁrst parent IgG (IgGantigen1) are N-terminally extended by an additional Fv fragment taken from a second parent IgG (FVantigen2, IgGantigen2). Two variants are achieved by employing either heavy or light chain as template (Fig. 1), and two types of each variant may be engineered. In variant 1, the heavy chain is the template and the variable domain of the second parent antibody (VHantigen2) extends the heavy chain of IgGantigen1 N-terminally. However, the light chain is crossed-over by inserting variable domain of IgGantigen2 (VΛntigen2) between VLantigen1 and the constant domain of the light chains (CL; Fig. 1: Type 1). The variable and constant domains of the light chains are connected by linkers L1 and L2, respectively. The variable domains and the constant domain of the heavy chains are connected by linkers L3 and L4, respectively. CODV-Igs are formed by oligomerization of two light and two heavy chains.

Swapping the Fv domains on both, heavy and light chains while maintaining the linker lengths results in type 2 (Fig. 1). Such constructs are distinct in three dimensions from the variant 2 constructs, which employ the light chain as template. Type 4 is obtained by N-terminally extending the light chains at FVantigen1 by VLantigen2 and inserting VHantigen2 between VHantigen1 and the first constant domain of the heavy chains (CH1; Fig. 1). Type 3 is derived from type 4 by swapping the Fv domains on both, heavy and light chains (Fig. 1).

We developed CODV prototypes using parental monoclonal antibodies directed against interleukins IL4 and IL13. Initial results indicated that identiﬁcation of appropriate lengths of four linking peptide stretches L1 to L4 requires signiﬁcant design (Supplements). Use of all-glycine linkers limited to a maximum of 20 residues, and with no consideration of the order of the two Fv domains, resulted in a search space of 214 constructs. To rationally limit the exploration of this search space, we employed a three-dimensional (3D) modeling strategy that used homology modeling of FvIL4 and protein-protein docking of the homology model of FvIL4 and the crystallographic models of both IL4 or FvIL13 (Fig. S1) at key steps. We identiﬁed four different spatial arrangements of FvIL4 and FvIL13 with suitable connectivity, and for which we expected no steric interference at the antigen binding sites either by the other Fv or the constant domains (Fig. 2). These arrangements suggest distinct sets of linker lengths of three to 15 residues, and give rise to either variant 1 or 2 (Fig. 1). They also suggest

![Figure 1](image_url)

Figure 1. CODV formats give rise to four distinct three-dimensional arrangements of Fvs and Fc. Each sketch represents half of a CODV-Ig that dimerizes by heavy chain pairing, resulting in tetramers of H2L2 stoichiometry. The two-dimensional sketches translate the different three-dimensional arrangements of the immunoglobulin domains. We adopt the following nomenclature for CODV biotherapeutics: in CODV-Igantigen1 x antigen2 and CODV-Fabantigen1 x antigen2, FVantigen1 is N-terminal on the heavy chain and consequently located between FVantigen2 and the first constant domain (Fc1) on the light chain no matter which engineering variant and linker lengths are concerned. We apply optimized all-glycine linkers of 7, 5, 1, and 2 residues for L1 through L4 in this work unless indicated otherwise.
that linker insertion at L4 or L2 is not obligatory in variant 1 or 2, respectively.

We confirmed these results experimentally by producing 150 CODV constructs using all-glycine linkers. We obtained type 1 and type 4 constructs with good yield and low aggregation propensity (Table 1, Table S1). Their binding affinity for both antigens is very well preserved. The median $K_D$ values of 6 pM and 60 pM for IL4 and IL13 binding, respectively, are as good as the $K_D$ values of the parental antibodies of 8 and 80 pM, respectively (Table 1, Table S1). Constructs of types 2 and 3 evince that inversion of the Fv domains results in equally well-behaved CODV-Ig. The optimal CODV-IgIL4 x IL13 prototype with linker lengths of 7, 5, 1, and 2 residues for L1 through L4, which was produced at 15 mg/L culture medium with 5% aggregation, binds IL4 and IL13 with a $K_D$ of 2.7 and 24.5 pM, respectively. We improved yield and aggregation propensity further by maintaining linker lengths and replacing the all-glycine stretches by sequences of arbitrary peptide composition or derived from natural CH1-CH2 domain transitions or VL-CL transitions of both k or λ light chains of IgGs. These constructs

![Figure 2](image_url) CODV format generates a self-supporting architecture that profoundly differs from DVD format. One- and two-dimensional schemes and crystal structures or three-dimensional models are depicted for the complexes CODV-FabIL4 x IL13/IL4, CODV-FabIL13 x IL4/IL13, CODV-IgIL4 x IL13/IL13, and DVD-FabIL12 x IL13/IL13. CODV-FabIL4 x IL13 of the crystal structures is a type 1 construct, CODV-FabIL13 x IL4 of the depicted initial models a type 4 construct.
are extremely well-behaved, with excellent yields, aggregation propensities, and KD values (Table 1).

The presence of either Fv does not affect the binding properties of the other Fv. Furthermore, antigen binding to Fv_{IL4} and Fv_{IL13} is independent and tetravalent (Fig. S2). Notably, we observe no positional, cooperative, or allosteric effects for CODV-Ig_{IL4 x IL13} in binding studies (Fig. S2).

**A self-supporting architecture for universal use**

The variation of linker lengths and sequences influences yield and aggregation propensity, but the vast majority of CODV constructs maintains antigen affinity at the level of the parental antibodies (Table 1, Tables S1 and 2). This suggests that almost all explored linkers permit proper Fv and Fc pairing, although to various extents. Once Fv and Fc domains are correctly paired, the CODV assemblies are stable.

We support this hypothesis by thermal stability studies and the determination of CODV-Fab crystal structures. We characterized thermal stability of CODV-Igs and CODV-Fabs directed against IL4, IL13, tumor necrosis factor (TNF), IL12/23, IL1β, insulin-like growth factor 1 receptor (IGF1R), and human epidermal growth factor receptor 2 (HER2) by differential scanning fluorimetry. Melting temperatures of the CODV-Igs and CODV-Fabs are in the typical range observed for natural IgGs (Tables S4A and B). Melting temperatures of CODV constructs directed against IL4 and IL13 are slightly reduced compared to the parental antibodies. Antigen order and linker composition do not affect melting temperatures significantly, however linker lengths affects melting temperatures slightly in the selected examples. Notably, only one melting temperature is observed in CODV-Igs, suggesting that melting of Fab- and Fc domains occurs concomitantly. We consider this observation as a hallmark of the circular self-contained architecture that functions as a self-supporting truss. Thermal stability of some CODV-constructs may be slightly reduced. We conclude that CODV architecture is overall as stable as natural IgGs due to proper Fv and Fc pairing.

We crystallized CODV-Fab_{IL4 x IL13} both in apo-form and complexed with IL4, and determined the structures at 2.7 and 2.6 Å resolution, respectively (Fig. 2, Table S4, Fig. 6). Both structures reveal a compact assembly formed by heavy and light chains. Both chains are properly dimerized as expected from the initial design and evinced by in vitro characterization. CODV-Fab_{IL4 x IL13} is stabilized by interactions at the dimer interfaces of both Fvs and Fc1. Linkers L1 to L4 are well defined in the electron density maps. Longer linkers are indeed required in the light chain to surround the heavy chain template for proper dimerization (Fig. 2). The structure of the complex reveals IL4 bound to Fv_{IL4}. The IL4 epitope of CODV-Ig_{IL4 x IL13} involves residues at the C- and N-terminal ends of helices H1 and H3, respectively, and part of loop H2-H3 (Fig. S4). Thus, IL4 interacts only with complementarity-determining region 3 (CDR3) of VL_{IL4} and CDR2 and CDR3 of VH_{IL4}.

Systematic maintenance of antigen affinities by both Fv domains at the level of the parental antibodies is a marked difference between CODV- and DVD-Igs, which we consider the most closely related bispecific IgGs (Fig. 2).26,27 The crystal structures of CODV-Fab_{IL4 x IL13} evoke reasons for maintenance of antigen affinities and independence of antigen binding in CODV format. The binding sites of Fv_{IL4} and Fv_{IL13} are about 65 Å apart, oriented in opposing directions, and very few interactions are observed between these domains or with Fc1 (Fig. 2, Fig. S5). Cooperative effects in antigen binding by IgGs are
proposed to be mediated by domain interactions between constant and variable domains. Residues of the second framework loop on the light chain may play a particular role. This loop corresponds to residues Lys157 to Lys163 in FvIL4 and Lys143 to Lys149 in FvIL13. These critical residues are not involved in direct domain contacts. Thus, the absence of cooperative or allosteric effects in surface plasmon resonance (SPR) measurements of antigen binding is coherent with the structural data.

No antigen binding site is obstructed by the presence of the other Fv or Fc1. Instead, the crossover architecture provides self-supporting constructs that allow for flexibility in the relative placements of the Fv and Fc domains. Comparison of the crystal structures of IL4-bound and antigen-free CODV-FabIL4 x IL13 suggests that the linkers maintain the relative placement of FvIL4 and FvIL13 tightly and allow for limited reorientation of Fc1 (Fig. S6). Access to the antigen binding sites is also unobstructed in the antigen-free structure. The comparison does not raise any concern on their accessibility even for large antigens. The well-maintained binding affinities for IL4 and IL13 of CODV-Igs with various linker lengths suggest that the self-supporting property is inherent to CODV architecture. Therefore, we conclude that the CODV format may be suitable for a wide variety of uses.

We evince universal use of the CODV-Ig format by constructs arbitrarily combining Fv domains from clinically applied monoclonal antibodies of different IgG germ lines. They bind antigens of various biological functions and sizes. They are directed against clinically validated targets, including TNF, IL12/23, IL1, HER2, and EGFR. All constructs are well behaved in terms of yield, aggregation propensity, and antigen binding compared to corresponding DVD-Igs (Table 1, Table S2).

CODV proteins are fully functional in terms of antigen binding. We also show efficient inhibition of cell signaling proteins by inhibition of IL4, IL13, or TNF signaling in cellular assays: IC50 values range from 33 to 54 pM, 0.9 to 4.5 nM, and 95 to 785 nM for IL4, IL13, or TNF signaling, respectively (Table 1, Table S2). Thus, CODV-Igs have the potential to satisfy therapeutic aims in clinical applications.

**Paratope orientations affect function**

We further proof the concept of their biological functionality by a T-cell dependent cytotoxicity assay probing CODV-Fabs directed against CD3ε or αβ-TCR expressed on primary human T-cells and CD19 expressed on NALM-6 cells (Fig. 3). This assay can only translate the biological efficacy of such "T-cell engager" molecules if both antigens on either T-cell or target cell are simultaneously engaged. DVD-FabCD3ε x CD19 and DVD-FabCD19 x CD3ε are generated with identical primary sequences for comparison. We observe highest and comparable cytotoxicity for CODV-FabCD3ε x CD19, DVD-FabCD3ε x CD19, and CODV-Fabαβ-TCR x CD19 with EC50 values of about 4 ng/ml. We note a positional effect in both formats when inverting Fv order: EC50 values are higher by 2 to 3 orders of magnitude for CODV-Fabs. The cytotoxicity of DVD-FabCD19 x CD3ε is lower by more than 5 orders of magnitude. In DVD-Fabs this positional effect could be explained by the positional effect on antigen affinity. However, the CODV format is not prone to a positional effect in antigen binding, yet we observe it in biological efficacy. Thus, we demonstrate the ability of these bispecific molecules to bind two membrane-bound antigens simultaneously, and show that the relative orientation in 3 dimensions of the antigen binding sites with respect to their epitopes in the biological environment is a critical property of multifunctional biotherapeutic proteins.

**CODV-Igs retain intrinsic IgG functions**

IgGs exert biological activities by interactions of their Fcs with Fc receptors and complement proteins such as C1q. To determine the effects of CODV architecture on Fc functionality, we quantified the affinity of CODV-IgIL4 x IL13 for FcRn and FcγRI by SPR. The affinity for FcγRI is very similar to isotype control IgG1IL4 while the affinity for FcRn receptors are slightly decreased by ∼2.5-fold (Table S3). We conclude that Fc functionality is a priori conserved in CODV-Igs.

FcγRs bind to natural IgGs at the transition of Fc1 to Fc2. Superposition and comparison of the crystal structure of IgG1 or the model of CODV-IgIL4 x IL13/IL4/IL13 with the crystal structure of the FcγR3/Fc complex indicate that the binding mode of FcγRs is unchanged (Fig. S8). Therefore, we assessed the ADCC functionality of CODV-IgIL12/23 x TNFα on Chinese hamster ovary (CHO) cells as target cells that express membrane-bound TNF by using Jurkat cells as effector cells that express FcγIIIIR. Antibody binding to FcγIIIIR with concurrent target cell engagement activates a NFAT driven luciferase reporter. Thereby, the assay reveals ADCC of the antibody. CODV-IgIL12/23 x TNFα and positive control IgG1TNFα induce ADCC (Fig. 4). Soluble TNF successfully competes with target cell engagement and inhibits ADCC (Fig. S10). CODV-IgIL4 x IL13/IL4 and CODV-FabIL12/23 x TNFα do not show ADCC due to lack of either target or effector cell engagement, respectively (Fig. 4). Thus, we evidence the potential of CODV architecture to achieve ADCC in vivo by tethering effector cells to target cells.

IgG antibodies elicit CDC by activating the classical pathway of the complement cascade via binding to C1 complex. Formation of ordered IgG hexamers as observed in the crystal packing of the structure of IgGgp120 increases affinity to C1 and thereby potentiates complement activation when viral or cellular surface
targets are recognized. Superposition of the CODV-IgIL4/IL4/IL13 model on the immune-competent IgGgp120 hexamer reveals that no steric hindrance for hexamerization by Fc2-Fc3 is introduced by the CODV architecture. Furthermore, the CDR regions remain accessible to antigens (Fig. S9). Thus, we assessed CDC activity of CODV-IgIL12/23 x TNFα on CHO cells expressing membrane-bound TNF. Complement-dependent cytolysis was detected by measurement of LDH release into the supernatant. CODV-IgIL12/23 x TNFα and positive control IgGTNFα induce CDC. Negative controls CODV-IgIL4 x IL13 and CODV-FabIL12/23 x TNFα do not elicit CDC due to lack of either target cell or complement binding, respectively (Fig. S10). Thereby, we show that the novel bispecific Ig format potentially increases therapeutic benefit by antigen-promoted CDC in specific indications.

**CODV-Ig binding to membrane receptors**

Finally, we asked how CODV-Ig could engage in cell-surface antigen binding, as in the dual receptor-targeting of HER2 and HER3 for which Phase 2 clinical studies are reported. Formation of HER2/HER3 heterodimers is a potent mitogenic signal that strongly depends on correct association of their transmembrane helices. Dual HER2/HER3 targeting aims at inhibiting their signaling, which can be achieved by simultaneously blocking HER2/HER3 dimerization and preventing their engagement in other heterodimeric complexes. We constructed homology models of CODV-IgHER2 x HER3/HER2/HER3 complexes including the transmembrane helices of HER2 and HER3. The models evince the possibility of concomitant binding of large antigens pairs at cell surfaces (Fig. 5, Supplements). They suggest that dimer association of transmembrane helices required for signaling may be blocked by suitably chosen paratopes of which the required orientation is maintained by the self-supporting CODV architecture (Supplements).

**Discussion**

The design of the CODV format is an example of applied research in drug discovery, yet it implicates innovation and its realization is based on atypical solutions from the point of modeling. Protein engineering usually involves site-directed mutagenesis, loop grafting, or creation of fusion proteins by connecting several domains by simple linkers. Intellectually and technically, these tasks are well mastered, in particular when experimentally derived 3D models support design. The design of fusion proteins also generally faces unperturbed folding of the individual domains as the only difficulty. More challenging designs are pursued in the fields of molecular evolution, protein folding and engineering. Here, protein design may aim at the creation of novel domain interfaces possibly with enzymatic function or adaptation of interfaces of oligomerization or repeating unit assembly that are perturbed when steric restraints are not properly taken care of in the design. Immunoglobulin domains and Fv fragments are versatile modules of proven value in biotherapeutics engineering. Our CODV design differs from all previously reported designs because we do not N- or C-terminally attach VL and VH domains to create linearly extended domain strings as in, for example, diabody (Db), single-chain variable fragment (scFv), or DVD-Ig formats. Instead, our cross-over design creates a circular self-contained architecture that gives rise to a self-supporting truss composed of Fv1, Fv2, and Fc1. The CODV design is thus akin to the design of circularly closed repeat architectures such as β-propeller and β-trefoil folds. Steric constraints must be matched to permit correct VH/VL pairing of Fv1, Fv2, and Fc1. In inappropriate linker lengths may result in VH/VL mismatch, leading to dimerization of DVD-Ig type with loss of target affinity as suggested by our initial constructs (Supplements). To rationally identify appropriate linker

![Figure 4](https://example.com/figure4.png)

**Figure 4.** CODV architecture retains intrinsic functionalities of natural IgGs such as ADCC and CDC. Immunoglobulins are tested on CHO cells as target cells that express membrane-bound TNF; effector cells are Jurkat cells that express FcγRIIIα and activate an NFAT driven luciferase reporter upon antibody-binding and cross-linking due to target cell engagement. Thus, ADCC activity of IgGTNFα, CODV-IgIL12/23 x TNFα, CODV-IgIL4 x IL13, CODV-FabIL12/23 x TNFα, and IgGIL4 is measured by activation of the Jurkat luciferase reporter translating ADCC (orange line and tilted square, redline and square, green line and triangle, blue dotted line and square, cyan dashed line and circle, respectively). Further data are reported in Fig. S10.
lengths and to limit the exploration of the search space of the 21 all-glycine linker mutants, we applied protein-protein docking of experimental and homology models of FvIL13, IL4, and FvIL4. Protein-protein docking is revealed as a sufficiently robust method to identify suitable domain arrangements for protein engineering, even if it is not reliable enough to routinely and unambiguously predict protein complexes or domain configurations as they are experimentally observed. Indeed, the modeling approach proved surprisingly efficient as we experimentally explored only ~0.08% of the search space and identified almost exclusively constructs with expected molecular weight that maintain parental target affinities.

The design of a robust, universally applicable bispecific therapeutic platform has to satisfy various parameters with regard to target antigen binding. Well-controlled affinity is one of these. Moreover, the proper balance of target affinity and avidity is critical to ascertain high efficacy with reduced undesired side-effects. As the CODV design guarantees unobstructed paratopes and stable VH/VL dimerization, it fulfils these requirements. Nevertheless, target affinity and avidity are not the sole parameters that drive efficacy. The 3D orientation and distance of the paratopes of bispecific agents can determine the biological effect. For instance, N- or C-BsAbsHER2 x HER2 in which the heavy chain of one IgGHER2 is N- or C-terminal extended by a second Fv-containing unit directed against another HER2 epitope have distinct cellular activities. Similarly, bispecific tandem scFv and tandem Fabs built with the two previously mentioned FvHER2 significantly differ in cellular assays. The circular self-contained architecture of the CODV format imposes defined paratope orientations by the lengths of linkers L1 to L4 that act like reins to adjust the antigen binding site directions. This possibility is unique among bispecific biotherapeutic formats because alternative architectures intrinsically allow for more flexible hinge-like movements of the Fv joining linkers. Thus, our results in the T-cell engager assays that systematically compare target antigen order in CODV- and DVD-Fabs clearly evince the importance of 3D paratope orientation at an even more subtle level compared to the cited
examples when target engagement aims at spatially tethering molecular or cellular units.

A universally applicable bispecific biotherapeutics platform should also provide uncompromised functionality of Fc domains to permit tailoring of pharmacokinetic properties and mechanism of drug action. Engineering interaction with FcRn gives control over the pharmacokinetic/pharmacodynamic profiles of biotherapeutic drugs, whether linear or non-linear pharmacokinetics will determine clearance.\textsuperscript{29,33,34,69,70} Thereby administration and dosing strategy can be tailored in function of disease requirements, which may differ widely in chronic and ad-hoc treatments. Systematic pharmacokinetic studies show that increased FcRn affinity leads to increased elimination half-life of IgGs; however, modulation of in vitro affinity does not result in predictive modification of the in vivo elimination profile. Affinity differences as much as 100-fold do not necessarily translate into statistically significant modification of elimination half-life, while the same level of in vitro affinity may give rise to significantly different elimination kinetics.\textsuperscript{71} FcRn contribute to IgG homeostasis by binding a histidine-rich site overlapping Fc2 and Fc3 of IgG.\textsuperscript{53} Superposition of the crystal structure of the FcRn/Fc complex and the model of CODV-Ig\textsubscript{IL4 x IL13} suggest that the binding mode of FcRn should be preserved in the CODV-Ig format (Fig. S7). No steric interference due to the presence of the Fv domains is obvious and binding of soluble antigens is likely to not interfere with FcRn-binding. As in vitro FcRn affinity is only minimally affected compared to isotype control, we anticipate that IgG-derived CODV-Igs are subjected to FcRn-dependent homeostasis. CODV-Fabs show the expected serum half-life of four to six hours in preclinical pharmacokinetic studies in mice.

Interaction with FcγRs or complement may be desired as part of the mechanism of drug action as in the trifunctional biotherapeutics catumaxomab and ertumaxomab or monoclonal antibodies daratumumab, 2F2, and 7D8.\textsuperscript{38,40,72-75} The circular self-contained CODV architecture with its voluminous Fab region could raise the concern that binding of FcRs or hexamerization which potentiates complement activation might be perturbed.\textsuperscript{40,51,76} Nevertheless, our experience in working with both CODV and DVD formats shows that the characteristics of the parental IgG of which the Fv domains are derived determine ease of production and purification in both bispecific formats. CODV and DVD proteins built with Fv domains of easily obtained Ig are well produced and purified, whereas bispecific proteins constructed with Fv domains of parental Ig that are performing worse regularly are more difficult to obtain in good quality and quantity. Indeed, the properties of employed Fvs dominate over the effect that Ig formats may have in production and purification of bispecific proteins. Therefore, we recommend systematic exploration of a large choice of parental Igs to choose the best Fv pair for the construction of bispecific Ig.

The close similarity of CODV proteins to natural IgG also facilitates exploration of the format in early stages of biotherapeutic drug discovery. Our results reveal that CODV architecture generates a structurally autonomous Fab equivalent with expected characteristics that can be employed accordingly. Furthermore, well-established techniques of IgG production and purification by affinity chromatography on protein A columns are applicable to CODV-Igs (Supplements). Protein A binds to the Fc of most human IgG at the transition of CH2 to CH3 and to Fv regions built with HC of sub-group III.\textsuperscript{80-84} Binding of protein A to IgG is concentration dependent and can be described by a bivalent binding mechanism.\textsuperscript{85-87} Superposition of crystal structures of Fc or Fab domains in complex with sub-domains of protein A on CODV-Ig models constructed with the type 1 crystal structure of CODV-Fab\textsubscript{IL4 x IL13} show that these binding sites are unobstructed in CODV-Ig except for steric interferences observed at the site of Fv1.\textsuperscript{81,86-90} Superposition of the Fab/protein A complexes on the initial type 4 model suggests unobstructed protein A binding sites on both Fv1 and Fv2. Thus, choice of linker lengths may influence purification of CODV-Igs, in particular when Fv domains with HC of sub-group III are employed.

In summary, CODV architecture is universally suitable for the development of bispecific multifunctional therapeutic proteins simultaneously targeting circulating or cell-surface antigens to elicit a curative effect. The format allows modification of the protein sequence to optimize properties such as antigen binding, solubility, or stability as it is state-of-the-art in monoclonal antibody design. It allows control of antigenicity by optimization of the framework regions and ensures efficient generation of structurally and functionally homogeneous protein. It is universally applicable with respect to targeting strategy, targeted antigens, integrated Fvs, isotype of Fc framework, and disease areas. The format unites the major advantages of alternative bispecific biotherapeutics to serve as a versatile starting point for fast-track biotherapeutic drug discovery. We currently apply it in disease areas as diverse as oncology, genetic, or infectious diseases, and expect major contributions to satisfy unmet medical needs.

**Methods**

Modeling and experimental work were carried out by state-of-the-art procedures under conventional conditions. In
Supplements, we report details and references on: design and analysis by molecular modeling; expression plasmid generation, protein production, and purification by affinity chromatography; biochemical characterization and size-exclusion chromatography; kinetic analysis of antigen- and FcR-binding by SPR; IC50 determination in cellular assays of IL4/IL13 or TNF activity; thermostability studies by DSF; protein crystallography; T-cell dependent cytotoxicity, ADCC, and CDC assays.

**Initial design of CODV proteins**

Homology models, CODV-Fab, and CODV-Ig models were constructed using programs of Schrödinger software package unless indicated otherwise by state-of-the-art procedures described in detail in Supplements.\(^{91-93}\) Initial CODV models are designed by the following 3D-modeling strategy (Fig. S1). Protein-protein docking of Fv\(_{IL13}\) and Fv\(_{IL4}\) was carried out with program ZDock employing standard settings in software package Discovery Studio.\(^{96-98}\) 2000 unrefined poses with 99 cluster centers and 478 singletons of the first screening round were exported for analysis in Maestro Package. The 99 cluster centers and the 478 singletons were visually inspected. Complexes of Fab\(_{IL13}\)/IL13 and Fab\(_{IL4}\)/IL4 were superimposed on each pose to reveal the possibility of proper N/C-terminal connectivity between Fvs and Fc, absence of steric interference at the paratopes potentially leading to loss in antigen binding, and to estimate linker lengths. Selected poses were merged with the structure of Fab\(_{IL13}\)/IL13 to construct homology models of CODV-Fab\(_{IL4}\) x IL13 with minimal, intermediate, and maximal linker lengths. Selected models of CODV-Fab\(_{IL4}\) x IL13 with minimal and intermediate linker lengths were checked for structural stability by molecular dynamics simulations at 298.15 K, 2 fs time steps, 100 or 500 ps trajectory, Surface Generalized Born implicit solvent model, and standard settings in program Impact. Neither structural instability nor any other structural problems were revealed by the molecular dynamics simulations. Detailed descriptions of construction and structural analysis of the final model of CODV-Ig\(_{IL4}\) x IL13/IL4/IL13 and homology models of CODV-Ig\(_{HER2}\) x HER3/HER2/HER3 are provided in Supplements.

The crystallographic coordinates are deposited in the Protein Data Bank (5FHX.pdb, 5HCG.pdb).

**Disclosure of potential conflicts of interest**

All authors are employees of Sanofi R&D.

**Acknowledgments**

A. S. acknowledges generous support by Drs J-Chr. Mozziconacci, D. Rinaldo, and J. Weiser. We also thank Dr D. Usener (Sanofi) for contribution of the TNF-expressing CHO cell line.

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