Engineered IgG1-Fc – one fragment to bind them all

Summary: The crystallizable fragment (Fc) of the immunoglobulin class G (IgG) is a very attractive scaffold for the design of novel therapeutics due to its quality of uniting all essential antibody functions. This article reviews the functionalization of this homodimeric glycoprotein by diversification of structural loops of CH3 domains for the design of Fcabs, i.e. antigen-binding Fc proteins. It reports the design of libraries for the selection of nanomolar binders with wildtype-like in vivo half-life and correlation of Fc receptor binding and ADCC. The in vitro and preclinical biological activity of selected Fcabs is compared with that of clinically approved antibodies. Recently, the great potential of the scaffold for the development of therapeutics for clinical use has been shown when the HER2-binding Fcab FS102 entered clinical phase I. Furthermore, methods for the engineering of biophysical properties of Fcabs applicable to proteins in general are presented as well as the different approaches in the design of heterodimeric Fc-based scaffolds used in the generation of bispecific monoclonal antibodies. Finally, this work critically analyzes and compares the various efforts in the design of highly diverse and functional libraries that have been made in the engineering of IgG1-Fc and structurally similar scaffolds.

Keywords: IgG1-Fc, Antibody engineering, Library design, Fcab

Introduction

Monoclonal antibodies (mAbs) undoubtedly belong to the most prominent therapeutics of the last two decades. Since 1986, more than 40 mAbs have been approved by the Food and Drug Administration (FDA) for the use in various diseases, including cancer, immune disorders, and infectious diseases, and many more are in clinical trials, with full-size IgG still representing the majority of these approved mAbs. However, based on the success of these drugs, several different antigen-binding formats such as antibody fragments or domains (i.e. antibody-based scaffolds) and non-antibody protein scaffolds have been investigated for their potential as binding molecules. The postulated advantage of such molecules is the reduced size that potentially leads to enhanced tissue penetration and facilitated production.

For example, three full-size antigen-binding fragments (Fab) of IgG have been approved by the FDA for clinical use so far [certolizumab pegol (1), ranibizumab (2), and
abciximab (3)]. At a considerable smaller size, single-chain variable fragments (scFv) comprise the variable domains of heavy and light chain connected by a polypeptide linker and therefore contain the complete binding sites of antibodies. It has been reported that scFvs can be readily expressed in various hosts like bacteria, yeasts, and plants (4–6). An advancement of this technology was the construction of bispecific T-cell engagers [BiTEs (7)], comprising two scFvs with specificities for CD3 and a target antigen expressed as one polypeptide chain. Dual-affinity re-targeting molecules (DARTs) follow a similar principle: As a further development of the diabody technology (8), a C-terminal disulfide bond covalently links two scFvs that are expressed as separate polypeptides to generate a more stable bispecific molecule (9). Xiao et al. reported the selection of molecules based on the isolated CH2 domain of IgG1 binding to the HIV-1 envelope glycoprotein (10). Another concept uses nanobodies, i.e. the single-domain antigen-binding fragments of camelid heavy chain antibodies, which, for example, have the potential for application in oral immunotherapy due to the increased resistance to extreme pH and proteolytic digest as compared to conventional antibodies (11, 12). Examples for non-antibody-binding scaffolds comprise, e.g. designed ankyrin repeat proteins (DARPins). DARPins are small, single-domain proteins derived from natural repeat proteins that can be engineered to bind diverse antigens (13). And finally, the immunoglobulin-like structure of the tenth type III unit of human fibronectin (10Fn3) has served as the basis for the engineering of one further novel binding scaffold (14).

The potential therapeutic value of these formats is, however, largely reduced due to the absence of the crystalizable fragment (Fc) and the resulting inability to trigger effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Accordingly, the in vivo half-life is limited due to the absence of the Fc-located binding site of the neonatal Fc receptor FcRn, and a lot of effort is necessary to overcome these drawbacks, which to some degree outweighs the advantage of the smaller size.

In 2009, Rücker and Wozniak-Knopp (15) reported the engineering of the structural loops of immunoglobulin constant domains to generate novel binding sites (Modular Antibody Engineering). Based on the observation that immunoglobulin-like domains are structurally conserved in the β sandwich core regions while at the same time exhibiting high variability of the loops (16), the three C-terminal loops of the CH3 domain of IgG1-Fc were engineered to bind diverse antigens in initial studies, yielding antigen-binding Fc fragments termed ‘Fcabs’. In this review, the therapeutic potential of such Fcabs and efforts in functional engineering as well as the engineering of biophysical properties will be discussed. There will be no focus on the engineering of individual antibody domains like monomeric CH2 or CH3 domains as scaffolds for the design of novel binders, as this topic has been reviewed recently (17).

**Structure of IgG-Fc**

The structure and function of human IgG-Fc has been described extensively. However, it is necessary to provide the background for discussion in the present review. Immunoglobulins of isotype G are the predominant antibody class in circulation and comprise two identical light (L) and heavy (H) chains forming a ‘Y-shaped’ structure (18). An IgG molecule can be dissected into two distinct fragments (Fab, Fc) that are responsible for the in vivo properties (Fig. 1): An antigen-binding fragment (Fab) is a heterodimeric protein composed of one light chain and the N-terminal half of one heavy chain. It encompasses two variable (V) domains (VH, VL) whose complementarity-determining regions (CDRs) form the antigen-binding site, and two constant (C) domains (CL, CH1) (Fig. 1). By contrast, the crystalizable fragment (Fc) is a homodimeric glycoprotein with one monomer consisting of two constant immunoglobulin domains (CH2, CH3) from the C-terminal half of one heavy chain (Fig. 1). Through interaction with its ligands [Fcγ receptors (FcγR)], C1q and FcRn] it mediates various immune effector functions and increases the half-life of the antibody molecule (19). The Fc and the two Fab
fragments meet at a central flexible segment, the hinge region, which plays an important role in the mediation between antigen recognition and effector functions and forms interchain disulfide bonds between the two heavy chains. X-ray crystallography of IgG1-Fc comprising amino acids 216–446 (20) (Fig. 1) normally reveals structure for

Fig. 1. Schematic representation of homodimeric human IgG1-Fc (PDB-ID 1OQO), generated using PyMOL, with the corresponding amino acid sequence. IgG1 is composed of two heavy and two light chains, with the Fab region carrying the antigen-binding sites and the Fc part mediating various effector functions. The homodimeric Fc part comprises the CH2 and CH3 domains, where β strands are depicted in green, and α helices and random coils are shown in gray. The C-terminal loops of the CH3 domain are colored in red (AB loop), orange (CD loop), and purple (EF loop). The asparagine at position 297 carries the glycan (NaNaFbi), graphically represented according to Anthony et al. (44). The amino acid sequence of the CH2 and CH3 domain of wildtype IgG1-Fc is shown and numbered according to Eu numbering system (20). On top of the sequence, the secondary structure elements of CH2 and CH3 according to the crystal structure (PDB-ID 1OQO) are shown.

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Immunological Reviews 270/2016
highly flexible (21 residues 238–260). In addition, the conformation of the hinge proximal region of the CH2 domain is shown to be ‘soft’, i.e. to be relatively mobile in comparison to the more defined structure observed at the CH2–CH3 interface.

Four distinct IgG subclasses differing in their heavy chains (and consequently in their Fc), exist in humans (hIgG1–4), and in mice (mlgG1, 2a, 2b, 3). This review will mainly focus on the structure–function relationships of the Fc of human IgG1 as a basis for engineering strategies for the design of novel therapeutic proteins. In the following, IgG–Fc and IgG1–Fc will always mean the basic homodimeric scaffold including two monomers, each being composed of the N-terminal hinge region followed by CH2 and CH3 domains (i.e. Thr225 to Lys446; for amino acid numbering and assignment of secondary structures, see Fig. 1).

A single N-linked glycan is attached to asparagine 297 (CH2 domain) of each heavy chain. The glycan has a complex biantennary structure (Fig. 1) and can vary by the addition of sugar residues to specific parts of the core structure. The latter is composed of N-acetylgalactosamine and mannose and can be modified by the addition of fucose, bisectioning N-acetylgalactosamine, and two arms defined by α1,3 and α1,6 mannose linkages (Fig. 1). The arms can be extended by the addition of galactose and sialic acid. There is a tremendous heterogeneity in the IgG–Fc glycan, with over 30 distinct glycans detected on circulating IgG in healthy individuals (22). The two Fc glycans are essential for the structural integrity of Fc (and IgG) by contributing mainly to the interface of the CH2 domains, where they face the center of the Fc with the α1,3 arm protruding into the cavity between the heavy chains and the α1,6 arm extending along the heavy chain backbone (26, 27). Crystal structures of IgG–Fc reveal a distinct conformation for the oligosaccharide resulting from multiple non-covalent interactions with the protein (23). This was confirmed by molecular dynamics (MD) simulations demonstrating that the glycans form more hydrogen bonds with the individual protein chains at the cost of glycan–protein interactions (28).

The thermodynamic parameters (obtained by differential scanning calorimetry) describing the unfolding of IgG1–Fc reflect these interactions, with the thermal unfolding of the CH2 domains showing a progressive reduction in stability with loss of sugar interactions while the unfolding of the CH3 domain is unaffected (29). Typically, IgG1–Fc exhibits two thermal transitions (best modeled using two sequential two-state transitions) with the lower temperature transition (Tm1 approximately 71°C) reflecting unfolding of the CH2 domain and the second transition (Tm2 approximately 82°C) unfolding of the CH3 domain at pH 7.4 (30). The latter underlines the importance of the extensive hydrophobic CH3–CH3 interface for the stabilization of IgG1–Fc. During unfolding, the CH2 domains behave as a single cooperative unit, and unfolding is reversible as long as the protein sample is taken to approximately 75°C at maximum, whereas unfolding of the CH3 domains is irreversible. Typically, the thermal stability of the Fc fragment decreases with decreasing pH as demonstrated experimentally (31) and by computational means (28).

Based on the importance of glycosylation for the conformational and thermal stability of IgG1–Fc, yeast surface display has been the method of choice to screen IgG1–Fc libraries for variants with desired properties (32). The advantage of yeast display over phage, bacterial, and ribosome display is the existence of a eukaryotic protein production machinery that is necessary for post-translational modifications (including glycosylation) and quality control of expressed proteins in the endoplasmic reticulum (ER) and the Golgi complex of eukaryotes (33). Also, in contrast to phage and ribosome display, yeast display selections usually include one or more flow cytometric sorting steps, facilitating quantitative analysis during selection. In addition, normalization for protein expression is possible by including expression tags (34).

Natural binding partners of IgG1–Fc

The most obvious advantage of IgG1–Fc over other antibody-derived or non-antibody binding scaffolds is its quality of combining all essential antibody functions except for antigen recognition, including binding to FcγRs. These may be expressed constitutively on hematopoietic cells (e.g. macrophages, eosinophils, neutrophils, natural killer cells, lymphocytes) and other tissues and may be induced or upregulated, differentially, on each cell type when exposed to cytokines or other activating agents (21). Stimulation of cells through FcγRs may result in the activation or deactivation of one or more of a variety of effector functions, including ADCC, CDC, phagocytosis, oxidative burst, release of inflammatory mediators, etc. (35). Due to the high sequence homology between the FcγR types and between the subclasses of IgG, spatial homology for interaction sites is found. In general, binding sites are located at the lower hinge and adjacent regions in the CH2 domain (36, 37) (Fig. 2).

A dynamic model of Fc–FcγR recognition was proposed in which oligosaccharide/protein interactions within the Fc
thermal stability, the two glycan chains of Fc are an absolute ligands (21). Besides contribution to the conformational and with distinct structures that recognize and bind individual protein generate an equilibrium population of conformers, constitutively and/or following induction or upregulation (43) and bind to IgG1 and IgG3 with similar affinity. While their external domains are highly homologous they transduce opposite signals (activating versus inhibitory) via their intracytoplasmatic domains. The binding site at the Fc is similar to that of FcγRI (21). Finally, FcγRIII (CD16) is a low-affinity receptor either expressed as an intrinsic (FcγRIIia) or glycosphingolipid-linked protein (FcγRIIib). Binding of natural killer (NK) cell-expressed FcγRIIia to the lower hinge region activates ADCC (37).

As mentioned above there is some variation in composition of IgG-Fc glycans in vivo and this may directly contribute to modulation of interaction (and affinity) with individual FcγRs classes, thereby mediating activating, inhibitory, or anti-inflammatory processes. Specific glycan forms have been associated with distinct immunological milieus (44). For example, increase in fucosylation and decrease in sialylation and galactosylation on the Fc glycan were observed during inflammatory conditions (45). There is an ongoing discussion whether these modifications are related to the expression of glycan-modifying enzymes like glycosyltransferases or whether other regulatory mechanisms are involved. Further studies are necessary to dissect the regulation of antibodies in vivo and this knowledge will also lead to the design and production of more efficient (glycoengineered) therapeutics, i.e. full-size mAbs or Fcabs, respectively.

Fig. 2 illustrates that C1q, the recognition subunit of C1 (i.e. the complex triggering activation of the classical pathway of complement) also binds at the N-termini of the CH2 domains. Similar to FcγRs, binding depends on the presence and mode of glycosylation at Asn297. It includes acidic and basic residues at CH2 but might also involve in vivo interaction with the CL domains of the Fab arms (46, 47). There are six heads on C1q, connected by collagen-like stems to a central stalk, and the isolated heads bind to the Fc rather weakly. Recently, it has been shown that antigen-binding on cell surfaces can facilitate the formation of IgG-hexamers and that these IgG-hexamers engage the headgroups of C1q (48). The resulting avidity effect increases the apparent affinity for C1q and triggers complement lysis. The IgG-hex-
amers are formed by non-covalent Fc–Fc interactions involving residues I253, H433, and N434. Mutation of these residues strongly reduces complement activation. Moreover, the authors also defined mutations in the IgG-Fc molecule that increased the formation of hexamers and thus resulted in improved activation of CDC. Thus, this study not only defined the molecular mechanism that triggers the classical pathway of complement but it also enabled the construction of Fc-mutants that activate the complement system more potently.

As mentioned above, one of the advantages of IgG-Fc-based therapeutic antibody fragments is the presence of a natural binding site for the neonatal Fc receptor FcRn (Fig. 2). FcRn mediates the transport of maternal IgG across the placenta in humans, thereby conferring humoral immunity to the fetus against antigens encountered by the mother (49). In addition, FcRn binds IgG with nanomolar affinity at acidic pH (≤ 6.5) in intracellular vesicles and releases it upon encountering the basic pH of the bloodstream (7.4). This recycling process prevents lysosomal degradation, ultimately resulting in prolonged serum half-life. The FcRn/Fc-binding interface spans a large surface area at the CH2–CH3 interdomain region. The center of the FcRn/Fc interface includes a hydrophobic core with surrounding salt bridges. At the Fc, the interface encompasses residues in the AB loop and the EF loop of the CH2 domain, as well as the G-strand of the CH3 domain (50).

Another highly specific receptor binding to the IgG-Fc region via its C-terminal PRYSPRY domain (Fig. 2) is the tripartite motif-containing 21 (TRIM21) protein. This cytosolic receptor recognizes antibody-opsonized pathogens and targets them to the proteasome through auto-ubiquitylation of E3 ubiquitin ligase. The neutralization mechanism of TRIM21 is thought to link the adaptive immune system with intracellular defense (51, 52).

Numerous other ligands bind to IgG-Fc, including proteins produced by microorganisms. The most prominent example is Staphylococcus aureus Protein A that binds with high affinity at the CH2–CH3 interface of IgG1 and IgG2 and is used for purification of mAb formats containing Fc (e.g. Fcabs).

**Generation of novel and improvement of existing functions in IgG1-Fc**

As outlined above, binding of ligands to IgG-Fc involves the N-termini of the CH2 domains as well as the CH2-CH3 interface (Fig. 2), whereas the C-termini of the CH3 domains are not affected. Each CH3 domain provides three C-terminal (structural) loops that can be diversified for the generation of novel antigen-binding sites: residues 358 to 362 (AB loop), residues 383 to 391 (CD loop) and residues 413 to 422 (EF loop) (Fig. 1). These loops correspond to regions with the most pronounced flexibility within IgG1-Fc (28). Upon engineering the loops, Fcabs, homodimeric Fc proteins with two potential antigen-binding sites in close vicinity, can be generated.

Wozniak-Knopp et al. (53) were the first to report the incorporation of antigen-binding properties in human IgG1-Fc. Five residues in each the AB loop (358–362) and the EF loop (413–415, 418–419) of the CH3 domain (compare with Fig. 1) were randomized using NNB degenerate codons and five additional random residues were inserted at position 415 to enlarge the potential binding surface. A 7.4 × 10^7 yeast surface display library was constructed and probed for binding to Protein A and FcγRI for comparison with surface-displayed wildtype IgG1-Fc. It was shown that a considerable amount of clones retained the binding to these proteins, suggesting structural integrity of the displayed molecules. Fc fragments binding to the extracellular domain of HER2 (an oncoprotein of the ERBB receptor family) were selected by fluorescence-activated cell sorting (FACS) using decreasing concentrations of the antigen. Next, the Fab exhibiting the highest binding affinity was matured, yielding the final clone H10-03-6, for which specific binding to HER2 at a K_D value of 8.6 nM could be determined by surface plasmon resonance (SPR) spectroscopy. Moreover, H10-03-6 was shown to elicit ADCC in an experiment involving a HER2-expressing cell line and primary human NK cells to an extent that was approximately 20-fold lower than for trastuzumab, a monoclonal antibody binding to HER2 which is applied in the treatment of certain breast cancers (54). Importantly, no adverse effect of loop engineering on the in vivo half-life of the molecule could be determined after injection of H10-03-6 or wild-type IgG1-Fc in BALB/c mice.

The correlation of FcγRIIIa-binding affinity and ADCC in Fcabs was further investigated by Kainer et al., who performed mutational studies to modulate ADCC potency. In a similar experiment as described above, HER2 overexpressing cells and NK cells were mixed and treated with variants of a HER2-binding Fc fragment carrying mutations that were previously reported to affect the affinity for FcγRIIIa. The authors concluded that known effects of the affinity-potency correlation can be assumed for the Fcab format (55).
with a special emphasis on the comparison with the clinically approved anti-HER2 antibody trastuzumab (56). Complementing the work described above, the authors demonstrated the simultaneous binding of FcγRIIIa and HER2 to H10-03-6 in an SPR-spectroscopic experiment as well as binding of FcγRIIIa to H10-03-6 on the surface of HER2-expressing SKBr3 cells. It was demonstrated that the epitopes that are recognized by trastuzumab and H10-03-6 overlap. Interestingly, a human tumor cell proliferation assay revealed that, in contrast to trastuzumab, H10-03-6 is not able to inhibit proliferation in a dose-dependent manner. However, when a preclinical in vivo tumor xenotransplant model using human HER2 expressing BT-474 cells was used to confirm tumor-killing via ADCC, a significant retardation of tumor growth could be determined which provided proof of the biological activity of the Fcab despite its obvious differences in functionality compared to trastuzumab.

A complementary study on the ADCC potency of H10-03-6 was published by Jez et al. (57), who expressed the Fcab in human cells as well as wildtype and glycoengineered plants to generate four different glycoforms of H10-03-6. The authors describe the crucial importance of the glycosylation pattern on ADCC activity, thereby confirming the applicability of concepts reported for full-size IgG to the Fcab format.

Recently, the discovery and preclinical activity of a novel HER2-targeting Fcab, FS102, was reported (58). Residues 358–362 and 413–419 in the AB and EF loops, respectively, were randomized to construct a yeast surface display library, which was screened for binding to the extracellular domain of HER2. The structural integrity of specific clones was confirmed by their uncompromised binding to anti-CH2, FcγRI, and Protein A. One resulting Fcab, FS102, contained a total of nine amino acid substitutions in both loops (compared to wildtype IgG1-Fc) but exhibited similar biophysical properties, meaning no major structural deviation between the two molecules. FS102 binds HER2 with high affinity comparable to that of trastuzumab and pertuzumab, but does not compete with either mAb in binding to the receptor, suggesting that it is targeting a different epitope. Interestingly, FS102 induces profound HER2 internalization and degradation and, finally, tumor cell apoptosis, which is an important mode of action for antibody therapeutics. The antitumor effect of FS102 in patient-derived xenografts correlated strongly with the HER2 amplification status of the tumors. At gene copy numbers of >10 HER2 per cell, superior activity of FS102 over trastuzumab or the combination of trastuzumab and pertuzumab was observed both in vitro and in vivo, and FS102 induced complete and sustained tumor regression in a significant portion of HER2-high patient-derived xenograft tumor models. The mode of action of FS102 is still under discussion but might be related to the structure of the Fcab and its two potential HER2-binding sites that are close together (20–40 Å) and relatively inflexible compared to the two typical IgG antigen-binding sites (120–170 Å). It is possible that the Fcab favors a more ordered and tightly packed interaction with the antigen and thereby initiates pronounced aggregation and internalization of the Fcab/antigen complexes. Binding of the Fcab might also favor a conformation of HER2 that is more susceptible to degradation.

Aside from the successful introduction of novel binding sites into IgG1-Fc, it was demonstrated that the C-terminal loops in the CH3 domains can also be engineered to generate pH-dependent binding between the Fcab and the respective antigen. As serum half-life controlled by FcRn is a major parameter to be considered for the applicability of therapeutic antibodies, engineering pH-sensitivity into the interaction of antibodies with their targets may also increase the clinical potential of these molecules. Increasing the affinity to the antigen in the plasma (pH 7.4) while simultaneously decreasing the interactions at acidic pH potentially reduces antigen-mediated clearance in the lysosome and therefore allows for administration of therapeutic antibodies at lower frequencies and doses. It was demonstrated that prolonged half-life of tocilizumab, a humanized antibody against IL-6 receptor, and antibodies binding to proprotein convertase subtilisin kexin type 9 can be engineered by subjecting several residues within the CDR loops to histidine scanning. The authors suggested that the antibody–antigen complex dissociates at endosomal pH and the antibody is salvaged from degradation by binding to FcRn which recycles it back to the cell surface (59, 60).

To apply this concept to IgG1-Fc, the HER2-binding variant H10-03-6 was used as model Fcab and a library was constructed by applying parsimonious mutagenesis to the regions coding for the binding loops of H10-03-6 in order to generate pH-dependent binding sites. The resulting yeast surface display library was subjected to alternating selections for binding at pH 7.4 and non-binding at pH 6.0 (61). Fcab variants could be selected whose interaction with HER2 was pH-dependent not only in the yeast display format but also when HER2-positive SKBr3 cells were titrated with solubleFcabs. Importantly, this effect was not caused by conformational changes, as shown by DSC and MD simulations. Only one variant contained a single His-substitution, but all
selected mutations were in the close proximity of existing histidines. The application of a surface display method for selections of pH-dependent binders in order to circumvent the labor-intensive histidine scanning approach was also demonstrated in a very recent study by Bonvin et al. (62). Here, scFv variants binding to the chemokine CXCL10 and enriched in histidine residues in the CDR-H3 were selected de novo from a phage library. Based on the selected lead clone, optimized libraries were constructed. When reformat-
tted into human IgG1, the isolated antibody 1A4 inhibited CXCL10-induced chemotaxis with an IC50 of 1.6 nM. In addition, the strong pH-dependency was evidenced by per-
foming a dose–response ELISA, where 1A4 exhibited a 167-fold lower affinity to CXCL10 at pH 6.0 than at pH 7.4.

Of course, not only can IgG1-Fc be engineered to obtain novel functionalities but also to improve existing functions. One example is the enhancement of the intrinsic property of ADCC elicitation. For instance, Lazar et al. (63) combined algorithms for computational prediction and high through-
put screening methods to selectively optimize the affinity and specificity for FcγRs. Designed Fc variants of the HER2-
binding monoclonal antibody trastuzumab exhibited ADCC enhancements over wildtype with the enhancement levels being proportional to the increase in affinity for FcγIIIα. Importantly, ADCC was observed for the engineered vari-
ants even with a cell line whose low surface levels of HER2 did not allow for ADCC detection after using wildtype trast-
uzumab. The authors concluded that especially for antibod-
ies that fail in inhibiting proliferation, enhanced engagement of the immune system by Fc engineering and thus mediated killing, i.e. by ADCC and CDC, could prove to be important.

Another very promising and constantly growing field in which the engineering of IgG1-Fc plays an important role is the generation of bispecific antibodies (bsAbs), which are able to simultaneously target two different antigens while at the same time maintaining important functions of a mAb. A number of different bispecific formats are currently in clinical trials or already approved for cancer therapy, with catu-
maxomab ( Fresenius Biotech), a mouse IgG2a and rat IgG2b hybrid antibody combining binding to CD3 and EpCAM that was approved in 2009, being a prominent example (64). Two other bsAbs include RG7221 ( Roche, Basel, Switzer-
land), a clinical phase II bsAb designed to bind Angiopoietin 2 and VEGF-A, and LY3164530 bsAb ( Eli Lilly, Indianapolis, IN, USA), currently being evaluated in clinical phase I and comprising binding sites for HER1 and c-Met (65–68).

The technologies proposed for the construction of the latter two molecules use different strategies to solve the light chain association problem. The CrossMAbCH1-CL technol-
ogy from Roche ensures correct pairing of the light chains by rearranging the CH1 domain of one heavy chain with the CL domains of the corresponding light chain (69), while bispecificity of the LY3164530 antibody is partly achieved by creating an orthogonal interface through mutations in V and L domains for the correct assembly of the different Fab domains.

The second prerequisite for the generation of bispecific antibodies is the enforcement of the correct heavy chain heterodimerization over wrong homodimerization, which requires modification of the Fc fragment. The knob-into-
holes (KH) approach mimics a key-locks system by intro-
ducing a bulky residue in one CH3 domain that favors bind-
ing to a small residue in the other CH3 domain (70). Atwell et al. (71) extended the KH technology by construc-
tion of a phage library of CH3 ‘hole’ mutants which were tested for binding to the T366W knob mutant and were able to select a heterodimer variant with improved thermal stability. To further stabilize and increase the purity of the heterodimeric Fc, an artificial disulfide bond was introduced by Merchant et al. (72). The latter development was exploited for the production of the above-mentioned bsAb RG7221.

Another approach represents the SEEDbody technology, which is based on the fact that the CH3 domains of human IgG and IgA do not dimerize (73). By using molecular modeling, the authors investigated interdigitating β-strand segments of IgG and IgA CH3 domains for the generation of complementary heterodimer contact surfaces.

One further technology to enforce correct dimerization includes mutations at the interface of the CH3 domains altering charge polarity of the heavy chain monomers. Two nega-
tively charged residues in chain A pair with two positively charged residues that were introduced in chain B (DD-KK variant) resulting in a high degree of heterodimer purity, but also a decrease in thermal stability of the CH3 domain (66, 74). Similarly, Strop et al. (75) identified mutations in the hinge region of human IgG1 and IgG2 that produce stabiliz-
ing ionic interactions. By combining these changes of the amino acid sequence with an additional mutation at the canonical position 409 in the CH3 domain, bispecificity was further enhanced. However, in this case both homodimers were first produced separately and then purified by affinity chromatography using protein A. Formation of heterodimers was induced by mixing both samples at equimolar ratios and
incubating them under reducing conditions at 37°C for 24 h, resulting in the most stably paired variant EEE-RRR for hIgG1. Moore et al. (76) identified residues in the CH3 interface promoting Fc heterodimerization by combining structural considerations with sequence information. Several variants were screened for the highest degree of heterodimerization, and an HA-TF variant was identified, where His and Thr are stabilized through a hydrogen bond and Ala and Phe act as key and lock.

Von Kreudenstein et al. described the heterodimer format ZW1 providing high thermal stability and 95% purity, with the residual 5% being present as monomeric, but not homodimeric CH2–CH3 subunits. By combination of in silico design and experimental screening, variants with high degrees of heterodimerization but low thermal stabilities were selected. Based on these initial results, modifications of the selected heterodimeric variants were designed toward increasing purity and stability. The leading format ZW1 carries four mutations on each CH3 domain located in the CH3–CH3 interface and exhibits wildtype-like Tm values (77).

In a very recent study, Choi et al. (78) combined electrostatic with asymmetric hydrophobic interactions and generated the EW-RVT Fc heterodimer. By introducing a disulfide bond between the CH3 domains they could further improve purity and thermal stability (79).

All of the above-mentioned approaches to the heterodimerization of heavy chains and the correct pairing of light chains aim at the production of full-size bsAbs providing one distinct specificity at each of the two Fab fragments. By contrast, application of the modular antibody technology would allow for the combination of these Fab-located binding sites with novel, artificial antigen-binding sites in the structural loops of constant domains. For example, the Fc of an existing mAb could be replaced with an Fcab to generate a so-called mAb2 that bivalently binds to one antigen via its Fab fragments and mono- or bivalently to a second antigen via the Fcab portion (Fig. 3).

**Engineering of the biophysical properties of IgG1-Fc**

The correctness of the overall fold of IgG1-Fc variants was confirmed in many cases. Under some circumstances it was necessary to improve the biophysical properties of Fcabs, either in advance by including stabilizing measures in the construction of libraries to make use of the full versatility of the scaffold or by ‘repairing’ functionalized, yet impaired, Fc fragments, which were mainly selected from early generation combinatorial libraries. Also, even though the Fc fragment is an intrinsically stable protein, the engineering of its properties could further accentuate the advantages over other non-antibody-based protein therapeutics.

A detailed systematic study on the effect that the engineering of the C-terminal structural loops of the CH3 domain of IgG1-Fc has on the biophysical properties of the Fc fragment was published by Traxlmayr et al. (80), who inserted the αvβ3 integrin-binding RGD motif in the respective loops. This motif, in the context of the heptapeptide

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**Fig. 3. Schematic representation of a hypothetical mAb2 [based on PDB-ID 1HZH (122)] and one isolated CH3 domain thereof.** Secondary structure elements of the antibody are colored in gray with the molecular surface of the CDR loops shown in green. Loop areas in the CH3 domains are displayed in red (AB loop), orange (CD loop), and purple (EF loop). The surface approximations of the three loops indicate the putative region for the generation of binding sites. © 2016 The Authors. Immunological Reviews published by John Wiley & Sons Ltd Immunological Reviews 270/2016
GCRGDCL, forms a cyclic and rather rigid structure as a consequence of disulfide bond formation by the flanking cysteines, resulting in increased interaction with integrins. Single-, double-, and triple-insertion variants (i.e. grafting of GCRGDCL into AB loop, CD loop, EF loop, AB+CD, AB+EF, or CD+EF) were expressed in Pichia pastoris and evaluated for the binding to soluble or cell-surface-expressed αvβ3 integrin as well as for the effect of the engineering efforts on the overall fold and the structural integrity of the protein. Expression levels determined for the variants did not differ significantly from those determined for the wild-type protein, except for the heavily mutated triple-variant, where grafting of the rigid circular motif into each of the C-terminal loops apparently resulted in severe misfolding and degradation. Aside from wildtype-like expression levels, all other constructs exhibited high purity and homogeneity and eluted as single peaks and at defined elution volumes in size-exclusion chromatography, suggesting the correct folding of the proteins and the absence of aggregates. Also, using electronic circular dichroism spectroscopy, the overall secondary structure content was determined for each variant. No significant changes were observed for AB- and CD loop variants, whereas engineering of the EF loop apparently affected the helical content of the protein. This effect was also reflected in the strongly decreased stability of EF loop variants as determined by DSC. In contrast to that, manipulation of AB and CD loop destabilized the protein to a lesser degree. As expected, simultaneous insertion in two loops had a more pronounced effect on protein stability than single insertions. Furthermore, the interaction with natural ligands was analyzed by SPR spectroscopy and biolayer interferometry: FcRn-, Protein A-, and FcγRIIIa-binding revealed to be wildtype-like, which again confirmed the structural integrity of the recombinant proteins.

The importance of biophysical properties of antibody fragments for large-scale production and/or therapeutic efficacy has been demonstrated in various studies. Willuda et al. (81) described the correction of an anti-EGP-2 scFv fragment which, despite its high affinity, did not enrich at tumor xenografts. By grafting the binding residues of this scFv onto the framework of a more stable fragment and identification and introduction of stabilizing mutations, a functionally improved variant could be generated for which tumor localization was observed.

In a different study, antibodies directed against the chemokine CCL17 were selected from a Fab phage library designed by Shi et al. (82), many of them raised from the VH1-69 germline gene family abundant in the human immune repertoire (83). Sub-nanomolar binders could be isolated, which, however, exhibited strong non-specific protein–protein interaction and low solubility. The authors suggested that the high hydrophobicity of the germline CDR-H2 leads to these unfavorable properties. By generating a library with randomized surface-exposed residues within CDR-H1 and CDR-H2 to a group of biochemically distinct amino acids, a panel of novel clones could be isolated comprising both high affinity and reduced non-specific interactions. Surprisingly, the overall hydrophobicity of the selected clones was not significantly reduced, but three residues turned out to be critical in terms of undesired protein–protein interaction. Clones exhibiting a mutation at position F51 to polar or charged residues as well as at position F54 to small polar residues demonstrated higher solubility. Furthermore, mutation of P52 suggested a structural change to the loop associated with higher stability. This study reveals the challenge in selecting for high affinity and good biophysical properties and demonstrates the importance of combinatorial library design, which we will discuss later in this review.

One further study describes a monoclonal antibody that neutralizes binding of angiopoietin 2 to its receptor in vitro and inhibits tumor growth in vivo (84). Even though the pharmacological activity could be demonstrated, production of the antibody coincided with heterogeneity of the preparations, rapid aggregation, and poor expression yields. Exchange of a susceptible surface-exposed cysteine by all other 19 amino acids yielded a threonine-variant that exhibited reduced proneness to aggregation, improved homogeneity, largely increased expression levels, retained activity and, interestingly, the midpoint of denaturation shifted by 11°C, corresponding to a strongly improved thermal stability. The authors stated that the engineering process resulted in the fulfillment of requirements necessary for large-scale production in order to provide amounts of material sufficient for clinical trials.

Considering the conclusions from the above-mentioned work, comparable efforts proved to be beneficial for the Fc scaffold. One successful attempt to contribute to the development of a ‘tool box’ that would help the correction of impaired Fc fragments was published recently (85). In this work, IgG1-Fc scaffolds with increased thermal stabilities were constructed by following a directed evolution approach. The authors implemented a novel method that allows screening for stabilizing mutations in proteins that exhibit an already high thermal stability and Tm values of up to 85°C. Two libraries of IgG1-Fc variants differing in their
mutation rates were constructed by error-prone PCR targeting the entire gene. These libraries were then expressed in the yeast surface display format and incubated at 79°C in order to denature those variants displayed on yeast that were not stabilized by mutation. Variants that were still conformationally intact were stained with fluorescently labeled structure-specific markers, either FcγRI or anti-CH2 antibody, and thereby tagged for sorting of the displaying yeast cell by FACS. As a consequence of heat denaturation of the yeast cells, plasmid DNA coding for the stabilized variants had to be isolated, followed by re-transformation of S. cerevisiae and construction of novel libraries, now being enriched in variants carrying favorable mutations. A total of four sorting rounds were performed to select for the most stabilized variants in the libraries which were eventually identified by sequencing of the isolated plasmid DNA. Biophysical characterization of 17 single, double, and triple mutants expressed in P. pastoris revealed that all of the variants exhibited increased thermal stabilities and wildtype-like binding to relevant ligands, i.e. FcRn, FcγRIIA, and Protein A. It was concluded that possible adverse effects of the artificial amino acid composition in the engineered loops might be counteracted by the introduction of the identified stabilizing mutations without negatively affecting the intrinsic functionality of the Fc fragment.

In addition, this original IgG1-Fc library pool generated by error-prone PCR and the selected libraries after one round of FACS were analyzed by high-throughput sequencing (86). For each amino acid position, the change in the mutation rate during selection was determined, indicating the tolerance to mutation at the respective position. As expected, selection for binding either to FcγRI or the anti-CH2 antibody resulted in reduced mutation rates. This was more pronounced in the CH3 domain reflecting the thermal denaturation pathway of IgG1-Fc with the reversibility of unfolding of the CH2 domain, as long as the CH3 domain remains natively folded. These data indicate a lower selection pressure for the CH2 domain, as only mutations either located in the binding site of the structure-specific markers or impeding the correct folding of the CH2 domain were eliminated. As both ligands that were used in this study bind to the CH2 domain, the changes in the mutation rates at positions in the CH3 domain were solely dependent on the impact of the respective side chain on foldability and/or stability, enabling the generation of a stability landscape of the CH3 domain. Importantly, positions that are evolutionarily conserved among different species were significantly less tolerant to mutation in these in vitro selections, validating the quality of the stability landscape. Furthermore, the experimentally derived tolerances to mutation correlated with the changes in the free energy of unfolding determined in silico. The results of this study not only revealed the sequence-stability relationship of an entire protein at single residue resolution but also proved to be an important tool for protein engineering, because library randomizations can be focused on mutation-tolerant regions of the protein. In addition, comparison of two selection experiments with different ligands (FcγRI and an anti-CH2 antibody) enabled the identification of their epitopes on IgG1-Fc.

In a follow-up project, this directed evolution protocol was applied to improve the biophysical properties of the HER2-binding Fcab H10-03-6 (87), which, even though the interaction with important effector molecules was wildtype-like, exhibited impaired biophysical properties in an initial characterization. In contrast to the method described above, mutagenesis was directed to the engineered loops only in order to minimize the overall changes the Fc fragment. An initial experiment revealed that the binding of H10-03-6 to HER2 was decreased after heat incubation, leading to the assumption that a correct fold is necessary for molecular recognition and that combination of heat shock and selection for ligand binding could be applied to simultaneously screen for improved thermal stability and retained affinity to the antigen. After heat incubation, the yeast displayed-library was either sorted for (i) binding to the antigen and to structurally specific ligands or (ii) binding to the antigen only. Enriched clones were expressed in P. pastoris and HEK293 cells and their biophysical properties were analyzed by SEC, ECD spectroscopy, and DSC. Stabilized variants resulting from the combined staining strategy (antigen and structurally specific ligand) exhibited higher thermal stabilities, which led to the assumption that this selection strategy should be favored. Importantly, all selected mutants were more stable than their parental clone H10-03-6. In addition, some stabilized Fcabs were less prone to aggregation after long term storage, showed more wildtype-like SEC elution profiles and the solubility of the Fcab exhibiting the highest thermal stability was largely increased when compared to the parental clone H10-03-6. The general applicability of this method to improve the biophysical properties of suitable proteins was concluded.

In addition, the differences in thermal stability of IgG1-Fc and Fcabs between the two expression systems, P. pastoris and HEK293, were analyzed. Generally, the high mannose glycan structures attached to Asn297 of the P. pastoris-produced Fc variants destabilizes the interface of the CH2
domains and therefore leads to lower Tm values. In a study by Schaefer and Pluckthun (88), significant differences between full-size IgGs produced in both expression systems revealed not only different temperatures of unfolding but also indicated differences in terms of aggregation susceptibility. Antibodies produced in P. pastoris were less prone to aggregate formation due to their mannose-rich glycan structure and the N-terminal residual EAEA extension, remaining from the α-factor pre-pro secretion sequence. Although the thermal stability of P. pastoris-produced Fc variants was lower, the corresponding SEC elution profiles were highly comparable with the HEK-produced counterparts (87), as was the stability-ranking of Fcabs in both expression systems. This clearly underlined that P. pastoris-produced Fc variants can be used to investigate the biophysical properties and binding characteristics of a panel of selected Fcabs.

A different approach to stabilize IgG1-Fc in order to provide a stable scaffold for the engineering of antigen-binding sites or other novel functionalities is based on the insertion of artificial disulfide bonds based on computational prediction (89). Selected cysteine-variants were expressed in P. pastoris and their biophysical properties were characterized, revealing that the thermal stabilities of two variants bearing novel intradomain disulfide bonds were largely increased while the overall structure remained wildtype-like. Combination of both novel disulfide bonds resulted in even higher thermal stabilities. Moreover, stabilization of the HER2-binding Fcab H10-03-6 could be achieved by the introduction of only one of the disulfide bonds.

In a second study, the observation that the C-terminus of the CH3-dimer closely resembles the C-terminus of the CH1-CL-dimer of the Fab fragment led to the assumption that the three C-terminal amino acids of the CH3 domains could be replaced by those of the CL domain (90). The resulting variant exhibited a wildtype-like elution profile in SEC, leading to the assumption that specific disulfide bonding was accomplished and no incorrect cross-bridging occurred. Thermal denaturation of the protein occurred at significantly higher temperatures compared to wildtype IgG1-Fc and this effect was observed to be further pronounced in a variant carrying both the stabilizing inter- and intradomain disulfides. Interestingly, more detailed analysis by DSC revealed a strong stabilizing cooperative effect that the CH3-located alterations had on the CH2 domain. The introduction of both the computationally and rationally selected mutants into H10-03-6 increased the thermal stability of the protein to be approximately wildtype-like while antigen-binding was retained. Those identified mutations could serve as a valuable tool for stabilization of single proteins or entire libraries.

In a similar study, Gong et al. (91) described the stabilization of a monomeric human IgG1-CH2 domain by the introduction of artificial, rationally designed disulfide bonds. For two variants whose expression levels were comparable to the wildtype domain and which were highly soluble, increased thermal and conformational stabilities were determined in heat and chemical denaturation experiments. However, only for the more stable variant a monomeric state was determined. Structural changes in this variant as a consequence of the cysteine mutations were analyzed by NMR, once again confirming the correct formation of the novel disulfide bond. Nuclear Overhauser effect spectra were recorded and showed that the flexibility of both the wildtype CH2 domain and the stabilized variant were rigid in the framework but highly flexible in the loop regions. Based on these findings, it was suggested that the CH2 domain as well as its stabilized variant can be applied as scaffolds for engineering antigen binders.

In summary, the methods described in this section can be applied in several ways: One possibility is the introduction of thus identified stabilizing mutations in naïve libraries in order to ‘prestabilize’ the scaffold and thereby minimize the risk of selecting an Fcab with impaired biophysical properties. The combination of several beneficial mutations exhibiting additive stabilizing effects could not only compensate for destabilization upon mutation of the structural loops but also further improve the characteristics of the resulting binders. Moreover, starting from a stabilized protein scaffold has been shown to promote evolvability by tolerating a wider range of mutations (92). In other words, a library based on a stabilized protein contains a higher fraction of correctly folded and therefore functional protein mutants. A more elaborate way of exploiting stabilizing effects of single or multiple point mutations would be the ‘repairing’ of existing binders as a reaction to negative effects of loop mutations, as was shown for the HER2-binding Fcab H10-03-6.

Optimization of library design

The previous chapters have clearly shown that IgG1-Fc is a very attractive scaffold for the design of novel binding molecules that possess all antibody functions at only one-third of the size of a full-size IgG1 molecule. Like many other therapeutic antibodies, antibody-based and non-antibody-based molecules, Fcabs are selected from combinatorial libraries that became an alternative to conventional
In this respect, the initial strategies that are pursued in order to provide a high degree of diversity, but also functionality, in libraries of different alternative binding scaffolds, are consistent. In this section, we will focus on these strategies and compare the library design efforts that have been made in the engineering of IgG1-Fc and other formats, especially the fibronectin type III scaffold, which, due to its immunoglobulin-like fold, is particularly interesting for comparison.

As mentioned above, the generation of diversity in alternative binding scaffolds cannot rely on the natural variation that is present in antibodies through somatic recombination and hypermutation during B-cell development. While in the course of this maturation process stable frameworks supporting diversity in variable domains are selected for, it is highly probable that diversification of one or several amino acid positions in alternative binding scaffolds impairs the overall protein fold to a certain degree. As mentioned above, in the case of Fcabs, it was shown that the introduction of an integrin-binding motif into each of the three C-terminal loops of IgG1-CH3 is possible while retaining the binding to generic ligands (80). However, overall folds of the proteins as well as thermal stabilities were impaired to some degree. The design of the first combinatorial libraries, i.e. the choice of amino acid positions in the C-terminal loops to be randomized, was based on the degree of evolutionary conservation and visual evaluation of the crystal structure of IgG1-Fc according to several criteria (solvent accessibility and structural independence of the respective amino acid side chains; generation of a coherent-binding surface) (15), and the applicability of this IgG1-Fc variant library was proved by isolating Fcabs binding to hen eggwhite lysozyme and CD20. Later, using an advanced library, it was shown that randomization of five positions in each the AB loop and the EF loop of IgG1-CH3 as well as insertion of five additional random amino acids in the EF loop, construction of a yeast surface display library and selection of IgG1-Fc variants binding to HER2 yielded well-expressing and biologically functional, yet slightly destabilized proteins (53). To circumvent this minor drawback already on the level of naïve libraries, two approaches have successfully been followed, which resulted in the construction of superior next generation libraries: First, prior to selections, naïve selections is more challenging and improves the resulting molecules’ developability, which for example is achieved by restriction to one stable framework or a limited number of consensus frameworks. The issue of immunogenicity is met by choosing framework and CDR compositions as close to the human germline as possible (93).

As the term ‘antibody-based’ is commonly used to describe formats that exploit the original binding site formed by the CDRs of either both or only one of the variable domains, antigen-binding IgG1-Fc, albeit derived from IgG1, will be classified as an ‘alternative binding scaffold’. In this respect, the initial strategies that are pursued in order to provide a high degree of diversity, but also functionality, in libraries of different alternative binding scaffolds, are consistent. In this section, we will focus on these strategies and compare the library design efforts that have been made in the engineering of IgG1-Fc and other formats, especially the fibronectin type III scaffold, which, due to its immunoglobulin-like fold, is particularly interesting for comparison.

Methods, i.e. the hybridoma technology. The selection of proteins with the desired binding properties from these libraries is in many cases accomplished by the application of various display technologies such as phage, ribosome or yeast surface display (93). Therapeutic IgG1 molecules are generated by selecting either scFv or Fab from combinatorial phage libraries, or full-size IgG from yeast or mammalian display libraries. The three main aspects of such libraries that are known to impact library quality are (i) design, (ii) the origin of sequence diversity, and (iii) the method of library generation, while library quality can be assigned to the library size, diversity, and the developability of the selected molecules, i.e. high affinity and good biophysical properties. Maximization of library diversity can be accomplished by increasing the functional size and improving the resulting molecules’ developability, which for example is achieved by restriction to one stable framework or a limited number of consensus frameworks. The issue of immunogenicity is met by choosing framework and CDR compositions as close to the human germline as possible (93).

The introduction of novel binding sites into non-complementarity-determining-region (CDR) loops in immunoglobulin-like domains of non-Ig proteins as well as immunoglobulin constant domains as already demonstrated in the context of Fcabs requires more elaborate considerations with respect to library design. While the lack of somatic hypermutation is, as is the case for CDR sequences of scFv or Fab fragments selected from combinatorial libraries, met by different affinity maturation strategies, the design of libraries for naïve selections is more challenging than in scFv or Fab libraries. First of all, there is little or no natural variation in the amino acid sequences of non-CDR loops and loop lengths are, in contrast to some CDR loops, mostly conserved. Information on typical amino acid compositions that can be drawn from the large pool of existing CDR sequences is not available for alternative scaffolds. And lastly, the knowledge of canonical CDR loop conformations is a design advantage that cannot be exploited in non-CDR loops as yet. Therefore, these favorable properties that are intrinsic to CDR loops have to be systematically investigated for non-CDR loops of Ig-derived and alternative binding scaffolds.

As the term ‘antibody-based’ is commonly used to describe formats that exploit the original binding site formed by the CDRs of either both or only one of the variable domains, antigen-binding IgG1-Fc, albeit derived from IgG1, will be classified as an ‘alternative binding scaffold’. In this respect, the initial strategies that are pursued in order to provide a high degree of diversity, but also functionality, in libraries of different alternative binding scaffolds, are consistent. In this section, we will focus on these strategies and compare the library design efforts that have been made in the engineering of IgG1-Fc and other formats, especially the fibronectin type III scaffold, which, due to its immunoglobulin-like fold, is particularly interesting for comparison.
identified for the generation of libraries of IgG1-Fc in order to minimize detrimental effects on the CH3 framework and increase the number of productive clones in the library (94). For this purpose, yeast surface display model libraries were constructed with distinct or overlapping regions randomized in the AB, CD, or EF loop of IgG1-CH3 and subjected to a protocol that involves the incubation at increasing temperatures of yeast suspensions induced for surface display and subsequent flow cytometric recording of the residual binding to conformationally specific ligands. Library denaturation curves were derived from the resulting mean fluorescent intensities and used for the determination of temperatures of half-maximal irreversible denaturation (T1/2) of entire yeast libraries, yielding a clear hierarchy of the distinct loop regions’ tolerance to randomization. IgG1-Fc libraries were re-designed based on these findings and are currently evaluated for their potential in the selection of Fcabs.

An approach to library design similar to the basic strategy pursued for Fcabs was published by Koide et al. (14), who identified the tenth type III unit of human fibronectin (10Fn3), an immunoglobulin-like β-sandwich protein, as a potent binding scaffold. Similar to the involvement of the N-terminal loops BC, FG, and C’E of IgG1-CH2 in the binding of the Fcγ receptors (39), 10Fn3 binds to integrin via the RGD motif in its FG loop. Initially, 10Fn3 library design was based on sequence analyses of several fibronectin type III domains as well as structural evaluation of the loop architectures. Selections using a library with five residues randomized in each the BC and the FG loop yielded a 10Fn3 variant binding to ubiquitin with an IC50 of 5 μM. However, this variant was less soluble at neutral pH than the wildtype 10FN3 protein and interacted with the column material in size-exclusion chromatography. In a follow-up study, it was suggested though that this negative side effect of mutation could be counteracted by the removal of unfavorable electrostatic interactions on the protein surface (95). Xu et al. (96) reported selections of 10Fn3 variants binding to TNF-α. The ‘master library’ applied in these selections consisted of an equimolar mixture of three sublibraries having either one, two, or all three of the N-terminal 10Fn3 loops randomized. Interestingly, all but one of the resulting TNF-α-binding 10Fn3 variants stemmed from the sublibrary having all three loops randomized, indicating the importance of diversity for the isolation of high-affinity binders. Again, the stabilities reported for two representative variants were decreased. In a follow-up study, Parker et al. (97) reported the significant loss of stability and solubility of VEGF-R2-binding 10Fn3 variants during affinity maturation, once more suggesting a trade-off between stability and affinity. The authors counteracted destabilization by structure-based site-directed mutagenesis, which also included the systematic reversion of randomized positions to their respective wildtype amino acids. Also, it was suggested to apply alternative designs of the apparently crucial DE loop of 10Fn3. All of these findings were based on the biophysical characterization of a limited set of 10Fn3 variants, which leads to the assumption that it would be possible to further enhance 10Fn3 library design (i.e. at which positions the N-terminal loops can be randomized) by applying the flow cytometry-based method developed for libraries of IgG1-Fc to this scaffold as described above, even though it is focused on stability and does not take into account the effects library design will have on affinity (94). If, on the other hand, results from one further study on the 10Fn3 scaffold by Lipovsek et al. (98) are taken into consideration, it can be expected that a higher number of randomized residues will increase the affinity of selected 10Fn3 variants while, as mentioned above, stability and solubility will be impaired. Consequently, if information on the importance of distinct regions for the overall fold and stability of the scaffold is made available in a systematic manner, as was done for IgG1-Fc, desired affinities can be approached by manipulating increasing numbers of distinct loop positions while at the same time being aware of the effect this will have on the biophysical properties of the scaffold.

Besides the application of modular antibody engineering to the C-terminal loops of IgG1-Fc-CH3, various other studies have described the introduction of novel binding sites to immunoglobulin constant domains: Xiao et al. (10) identified HIV-1 inhibitors based on isolated IgG1-CH2 by randomizing the longest N-terminal loops in the domain, i.e. BC and FG. An interesting design feature was the addition of a glycine residue at the C-terminal end of each loop to provide flexibility and favor the accommodation of amino acid alterations necessary for stability and antigen recognition. However, as the stability of native CH2 is relatively low, the solubilities of three HIV-1-binding ‘nanoantibodies’ selected from this library were reported to be poor. To overcome these issues, the isolated CH2 domain was stabilized by introducing an additional disulfide bond and removing seven N-terminal residues (91, 99, 100). Starting from this scaffold, a library was constructed that comprised randomized BC and DE loops and a CDR-H3 from the HIV-1 gp120-binding VH m36 grafted in place of the FG loop (101). While one of two clones selected from this library

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Immunological Reviews 270/2016
interacted non-competitively with an HIV-1 neutralizing epitope and FcRn, the second was not further characterized due to its aggregation proneness, leading to the authors’ conclusion that further improvements to the library design would be necessary to develop IgG1-CH2 as a scaffold for the development of novel therapeutics.

Length variation of CDR loops is another important factor in the natural generation of diversity. Among the six CDR loops, H3 is the most diverse, both in terms of sequence and length. In a study on the clustering of antibody CDR loop confirmations, North et al. (102) assigned between two and eight different loop lengths to L1, L2, L3, H1, and H2, and 20 different lengths to H3, ranging from 5 to 26 residues. This length variability is introduced by imprecise joining during the combinatorial rearrangement of VH, DH, and JH genes in the course of B-cell maturation (103) and is considered to be a main contributor to the recognition of diverse antigens, as H3, given by its location in the center of the antigen-binding site, potentially controls the relative positions of VH and VL and affects the flexibility and cavity size of the paratope (104).

Consequently, in order to mimic this crucial aspect of the development of antigen specificity by the immune system, loop length variation in the antigen-binding sites of alternative scaffolds is an important factor in the generation of specific, high-affinity interactions. In the process of development of the Fcab scaffold, several steps have been made toward a loop elongation strategy that would allow for the selection of variants binding to diverse antigens while at the same time the intrinsic stability can be retained to a satisfactory degree. As mentioned above, the initial design for variant libraries of IgG1-Fc included the insertion of five additional residues at the N-terminal part of the EF loop, with this loop elongation aiding in the selection of an Fcab binding to HER2 with a $K_D$ value of 8.6 nM (53). However, mutation and insertion negatively affected the biophysical properties of this Fcab (87). Therefore, one further aspect of the detailed analysis of the C-terminal loops of IgG1-CH3 was the systematic insertion of five additional random residues at different loop positions according to a sliding window (94). $T_{1/2}$ values were determined for the resulting yeast surface display libraries as described above and used for the comparison of the tolerance of different loop positions to insertion. The results from this study, as well as the identification of sites of natural insertion from phylogenetic analysis of IgG1-CH3 from different species, were also considered for the design of novel variant libraries of IgG1-Fc. As mentioned above, the evaluation of the effect of these design features is part of current investigations. Furthermore, findings from a study that dealt with the identification of stabilizing point mutations in IgG1-CH3 by using a directed evolution approach were combined with rational considerations (85, 105). In order to generate stabilizing stem regions that would allow for loop elongation, point mutations were introduced at two intrinsically stable motifs in the EF loop. The central part of this loop is constituted by an arginine residue forming a salt bridge with a glutamic acid in the CD loop, and a tryptophan residue that has been shown to be crucial for protein stability by contributing to the packing of the hydrophobic core. At the C-terminus of this RW motif, a glutamine residue was replaced by a stabilizing leucine residue. At the N-terminus of the adjacent F-strand, a serine between a phenylalanine also contributing to hydrophobic packing and a cysteine forming the intradomain disulfide bond was replaced by a stabilizing threonine.

Yeast surface display libraries including or not including these stabilizing stem regions and carrying increasing numbers of additional residues in between them were constructed, and their stabilities were evaluated by using the yeast surface display-based method described above (94), showing that, indeed, a largely increased tolerance to loop elongation was achieved by this stabilization approach, with the potential of such libraries for the selection of Fcabs binding to diverse antigens being yet to be looked into.

While the work on the design of libraries of IgG1-Fc to date has focused on the question of how enhanced diversification, both in terms of site- or region-specific randomization and loop elongation, affects the overall fold and the biophysical properties of the scaffold, the trade-off between this focus and its effect on Fcab affinity is still under investigation. However, this has been addressed for other alternative formats, including the above-mentioned immunoglobulin-like $^{10}$Fn3 scaffold, which is why the results of the underlying studies should be of interest in this context and a link can be made to ongoing and future projects dealing with IgG1-Fc. Koida et al. (106) performed basic experiments on the potential for elongation of all the loops in the $^{10}$Fn3 domain, thereby providing some first information on the tolerance to insertion of alanines, which, of course, does not reveal the effect of full randomization. This was further investigated by Hackel et al. (107), who diversified all three N-terminal loops of $^{10}$Fn3 both in length and composition. Four different lengths were chosen for each loop based on sequence analysis of $^{10}$Fn3 from different species, and NNB degenerate codons were used to incorporate all 20 amino acids. Three FACS sortings of the
naïve library against lysozyme were performed. The output of this naïve selection was diversified by loop shuffling and error-prone PCR and sorted twice, followed by another four rounds of this procedure, yielding $^{10}$Fn3 variants of single digit picomolar affinity and high sequence diversity, having accumulated a considerable number of beneficial framework mutations. Interestingly, further mutational studies on the DE loop of one particular high-affinity $^{10}$Fn3 variant revealed that this loop can be either engineered to improve affinity or stability to a considerable degree. These results underline once more that library design for scaffolds based on immunoglobulin and immunoglobulin-like domains must undergo adjustments based both on the systematic evaluation of naïve libraries and selection outputs in order to efficiently approach an optimum balance of stability and affinity of relevant variant proteins.

The degree of CDR sequence diversification in combinatorial libraries of antibody-based alternative scaffolds, i.e. the usage of the amino acid repertoire, follows various different strategies. While naïve libraries provide natural diversity by combination of functional V-gene segments isolated from the lymphoid tissues of non-immunized donors [e.g. CAT 1.0 and CAT 2.0 libraries of Cambridge Antibody Technology (108, 109)], synthetic libraries such as the HuCALs (Morphosys) offer any desired amino acid compositions in the CDRs (110, 111). In general, it was observed that, despite the hypervariability of CDR sequences, there is a bias of functional CDRs to certain amino acid types, most of all tyrosine, glycine, and serine (112). Fellouse et al. showed that while scFv variants binding to some antigens at nanomolar affinities were selected from phage display libraries providing only a binary amino acid repertoire (i.e. tyrosine and serine), selections of binders from the same libraries to other antigens yielded affinities only in the micromolar range (113–115). Modifications of these libraries included an increased chemical diversity in CDR-H3 and the consideration of non-paratope residues potentially important for CDR conformation (116).

As mentioned above, Morphosys’ HuCAL Fab libraries are fully synthetic, and they were developed over the course of several years (110, 111). The latest version, named HuCAL PLATINUM, offers, among other features, length variation in CDR-H2 and CDR-H3, and length-dependent amino acid frequencies as observed in the analysis of rearranged sequences, which was realized by using a large set of different trinucleotide mixtures. In principle, a similar approach is potentially valid for the construction of variant libraries of IgG1-Fc. Naturally, while the alignment of thousands of VH and VL sequences was used to generate information on the amino acid distributions in CDR loops, it cannot be estimated how, for example, binary codes or natural distributions in CDRs would influence the efficient isolation of well-developable Fcabs binding to diverse antigens at high affinities. Nevertheless, Hackel et al. investigated the effect of full or restricted diversity in $^{10}$Fn3, which, again, can be a valuable source of information for the engineering of IgG1-Fc due to its immunoglobulin-like fold (117, 118). In these studies, the sequence-function landscape of the $^{10}$Fn3 scaffold was approached by comparing various amino acid compositions in the N-terminal loops, leading to important conclusions: First, it was shown that maximal amino acid diversity is more beneficial for the selection of antigen-binding $^{10}$Fn3 variants than a binary tyrosine/serine code as described above. And second, wildtype conservation at positions potentially important for structural integrity, as well as amino acid distribution biases toward the natural occurrence in CDRs made clear that both strategies were valuable for an efficient selection of binding $^{10}$Fn3 variants. Consequently, while the identification of positions in IgG1-Fc-CH3 at which conservation of wildtype amino acids improves the biophysical properties of selected variants has been accomplished (86, 94), the application of tailored amino acid distributions as described above is a promising approach to be investigated in the future.

Conclusion

Over the past 30 years, therapeutic monoclonal antibodies and antibody-based alternative formats have been successfully developed, first by using the hybridoma technology and later by selection from combinatorial libraries. In this review, we introduced the potential of IgG1-Fc as a promising scaffold for the generation of alternative protein therapeutics. By applying the modular antibody engineering technology, novel binding sites for theoretically any desired antigen can be introduced in the C-terminal loops of the CH3 domains to create Fcabs, antigen-binding Fcs. In this way, the Fc is engineered to unite all important functions normally conveyed by full-size IgGs, i.e. (i) the mediation of effector functions through binding to FcγRs; (ii) contribution to a long half-life in serum through binding to FcRn, and (iii) (novel) binding to an antigen. FS102, a HER2-binding Fcab that has recently entered the clinic, is believed to accelerate aggregation and internalization of the antigen/
Fcab complexes as a consequence of the distinct Fcab structure, with pharmacokinetics not being impaired by the sequence changes in the CH3 domain.

In some cases, as has been described for other alternative scaffolds, the engineering process might negatively affect the biophysical properties of IgG1-Fc. The current article summarizes a set of methods, mostly based on directed evolution and yeast surface display, which provide a ‘toolbox’ for the correction of impaired biophysical properties of proteins in general. We also reviewed the various approaches to modifying IgG-Fc for the production of bispecific antibodies and described the highly promising concept of constructing mAb²’s, monoclonal antibodies with one specificity in the two antigen-binding sites of the Fabs and one additional specificity at the C-terminus of the Fc, which again can be accomplished by modular antibody engineering.

Finally, this review provides comments on potential further developments of libraries of IgG1-Fc. Loop position- and loop region-specific information has been obtained that will aid in the construction of libraries of high quality, and studies dealing with the modification of the architecture of the putative Fcab binding sites by loop elongation have been performed. Many approaches that have proved successful in the context of other alternative scaffolds, especially those targeting tailored amino acid distributions, can be used as models for the future investigation of libraries of IgG1-Fc, which will ultimately lead to a further accentuation of the intrinsically favorable properties of FCabs.

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