Despite the enormous success of antibody-based therapeutics for the treatment of a variety of diseases, research efforts to improve their clinical efficacy continue. One avenue being explored is the engineering of new antigen binding sites to permit co-engagement of two distinct targets. Such engineered antibodies are commonly referred to as bispecifics, and a wide variety of formats have been described in references 1 and 2. Co-target antigens can include two targets believed to be causal in the pathology of a particular disease, e.g., two cytokines or growth factors.3-5 Alternatively, the co-target pair may be a cell surface antigen and an immune receptor such that a novel “effector” mechanism can be built into the antibody, beyond those mediated naturally by the Fc region.2

In the 1980s, bispecific antibodies were made by fusing two cell lines that each produced a single monoclonal antibody (mAb).6 Although the resulting hybrid hybridoma or quadroma did produce bispecifics, they were only a minor population and extensive purification was required to isolate the desired antibody. Antibody fragments provided an engineering solution to this problem; because they lack the complex quaternary structure of a full-length antibody, multiple variable regions can be linked in single genetic constructs. Antibody fragments of many different forms have been generated, including diabodies, single chain diabodies, tandem scFvs and F(ab’)_2 bispecifics.2,7 While these formats can be expressed at high levels in bacteria and, arguably, may have benefits due to their small size, they suffer from poor half-life in vivo and can present manufacturing challenges related to their production and stability. For example, the rapid clearance of some fragment-based bispecifics requires that they be infused continuously via a portable pump over one to two months.8 The principal source of these limitations for fragment formats is the lack of an antibody Fc region with its associated structural and functional benefits, including large size that precludes renal filtration; high stability; binding to various Fc ligands, one of which maintains serum persistence (the neonatal Fc receptor FcRn) and binding to proteins A and G, which facilitates large scale purification.

Recent work has attempted to address the shortcomings of fragment-based bispecifics by engineering a second antigen binding site into full-length antibody-like formats.5,9-12 The presence of an Fc region in theory provides these formats with the developability and pharmacokinetic properties of standard IgG mAbs. However, because these constructs build new antigen binding sites on top of a homodimeric constant chain, binding to the new...
antigen is always bivalent. This consequence may pose a constraint depending on the co-targeting goal.

For many immune receptors, cellular activation is accomplished by cross-linking of a monovalent binding interaction. The mechanism of cross-linking is typically mediated by antibody/antigen immune complexes, or via effector cell to target cell engagement. For example, the low affinity activating Fc gamma receptors (FcγRs) such as CD16 (FcγRIIA) and CD32a (FcγRIIA) that mediate cellular killing bind monovalently to the antibody Fc region. While monovalent binding does not result in cellular signaling, upon effector cell engagement with the target cell, receptors are cross-linked and clustered on the cell surface, leading to activation.13 On T cells, CD3 activation occurs when its associated T-cell receptor (TCR) engages antigen-loaded MHC on antigen-presenting cells in an avid cell-to-cell synapse.14 Major histocompatibility complex (MHC) on antigen-presenting cells is always bivalent. This consequence may pose a constraint depending on the co-targeting goal.

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Bivalent antibodies targeting major histocompatibility complex (MHC) on antigen-presenting cells in an avid cell-to-cell synapse.14 Bivalent antibodies targeting CD3 can elicit massive cytokine release, and the consequent toxicity has presented challenges for the development of anti-CD3 antibodies as drugs;15,16 in contrast, monovalent binding to toxic ing CD3 can elicit massive cytokine release, and the consequent toxicity has presented challenges for the development of anti-CD3 antibodies as drugs;15,16 in contrast, monovalent binding of CD3 in Fabγ7,18 and bispecific19 formats generates much lower levels of T-cell activation. For bispecifics, a consequence of this biology is that bivalent cross-linking of receptors can lead to non-specific activation of an effector cell in the absence of target cell.

Thus, when the therapeutic goal is the co-engagement of an immune receptor, the desired binding may be monovalent rather than bivalent. This mode is incompatible with the majority of current full-length bispecifics. We describe an engineering solution to this problem that utilizes a heterodimeric Fc region to enable a single additional variable region to be built monomerically onto an antibody. Our new bispecific format, which we refer to as mAb-Fv, enables the simultaneous bivalent and monovalent co-engagement of distinct target antigens.

Results

Engineering of heterodimer-selective Fc variants. We designed variants that promote Fc heterodimer and discourage homodimer formation using structure- and sequence-based approaches. Structural calculations explored energies of paired variant combinations at residues that interact across the C\textsubscript{H3} dimer interface. Pairs that were predicted to have lower energy in the heterodimer (both variant amino acids) relative to the two homodimers (variant amino acid at one position and the native amino acid at the other) were incorporated into the experimental library. Design also benefitted from an analysis of the interfaces of human C\textsubscript{H1}/C\textsubscript{K} and human C\textsubscript{H1}/C\textsubscript{\lambda}, which are naturally heterodimeric. The primary library contained 41 paired variants.

We constructed Fc variants in the context of an Fc/scFv-Fc system (Fig. 1A and B) in order to screen for their effect on hetero and homodimerization. DNA constructs were designed encoding two different immunoglobulin polypeptides: (i) an Fc (Hinge\textsubscript{C\textsubscript{H2}}-C\textsubscript{H3}) and (ii) an scFv-Fc (V\textsubscript{H}-linker-V\textsubscript{L}-Hinge-C\textsubscript{\lambda}-C\textsubscript{\lambda}). Here the two different C\textsubscript{H3} domains, C\textsubscript{H3}\textsuperscript{\alpha} and C\textsubscript{H3}\textsuperscript{\beta}, represent the designed variant pairs in the library. Cotransfection and expression of these two polypeptides results in three possible dimers, each composed of a unique number of Ig domains and differing molecular weights (MW), thus enabling measurement of each species using gel electrophoresis. Pairs of Fc and scFv-Fc constructs were cotransfected into HEK293E cells\textsuperscript{20} for expression, and the resulting protein was purified from the supernatant using protein A affinity chromatography.

We visualized proteins electrophoretically under reducing and non-reducing conditions. Under reducing conditions two bands were observed (data not shown), corresponding to Fc monomer and scFv-Fc monomer. Under non-reducing conditions three bands were observed (Fig. 1C and Fig. S1), corresponding to Fc homodimer, Fc/scFv-Fc heterodimer and scFv-Fc homodimer. Quantification of the bands enabled identification of variant pairs that stabilized heterodimers relative to homodimers (Fig. 1D). As expected, native IgG1 Fc resulted in an unbiased distribution of pairs, with ~50% heterodimer and ~25% of each homodimeric species. Variants shifted this distribution by varying extents, with the best variant pairs S364H-Y349T (H-T) and F405A-T394F (A-F) resulting in 61–71% heterodimer formation (Fig. 1C and D). Structural models of these variants were generated by computational methods\textsuperscript{21} (Fig. 2).

In a subsequent round of design and screening, we combined 19 lead single variants in a library of 22 double substitution variants (four substitutions in the heterodimer). Combination variants increased the heterodimer population up to 83%, with the best performers being S364H/T394F-Y349T/F405A (H-TA) and S364H/F405A-Y349T/T394F (HA-TF) (Fig. 1C–E). The thermal stability of the HA-TF variant compared with the alternative HA-HA and TF-TF homodimeric combinations was examined using differential scanning calorimetry (Fig. 1F). The HA-TF heterodimer (T\textsubscript{m} = 69°C), although less stable than native IgG1 Fc (T\textsubscript{m} = 72, 83°C), was found to be more stable than the HA-HA and TF-TF homodimers (T\textsubscript{m} = 61, 57°C). Based on its high heterodimer formation (83%) and relatively high thermal stability compared with that of the competing homodimers (ΔT\textsubscript{m} = 8, 12°C), the HA-TF variant was selected as the lead for our bispecific construct.

Following the selection of the HA-TF variant as our lead, we benchmarked its heterodimer formation against two other heterodimer-selective Fc variants found in the literature. We also examined the effect of perturbing the transfection ratio of the Fc and scFv-Fc constructs as a model for the case of unequal expression of the two chains. Variant pairs T366S/L368A/Y407V-T366W\textsuperscript{22} (SAV-W) and K392D/K409D-E356K/D399K\textsuperscript{23} (DD-KK) were constructed in the Fc/scFv-Fc system, expressed and purified as described above. Proteins were visualized electrophoretically under non-reducing conditions. Heterodimer yield of the lead HA-TF variant (89%) was similar to that of the SAV-W (85%) and DD-KK (89%) variants (Fig. S2) and was sensitive to transfection ratio, as previously observed.\textsuperscript{22,24} These results indicate that our lead HA-TF variant is performing in a comparable manner to other published variants.

Design of the mAb-Fv format. Our bispecific design goal was to build monovalent binding to a second antigen into a full-length antibody format. We used our engineered HA-TF variant pair to promote heterodimerization of the Fc and thereby enable fusion of V\textsubscript{H} and V\textsubscript{\lambda} domains to the C-termini of two
distinct heavy chains. This construct, which we call mAb-Fv, is essentially a full-length antibody with an additional C-terminal Fv region (Fig. 3A). The two identical N-terminal Fv regions are those of a typical full-length IgG, and thus provide bivalent antigen binding. In contrast, the engineered C-terminal Fv is monovalent. We reasoned in our design that the C-terminal VH/VL pairing may contribute further to the heterospecificity of the heavy chains, although we did not explore the possibility of this benefit in a controlled way.

As test systems of the mAb-Fv, we targeted the NK cell activation receptor CD16 and the T-cell activation receptor CD3 as second antigens. VH and VL variable domains of the murine anti-CD16 antibody 3G825 were fused to the C-termini of hetero-Fc variant heavy chains of the anti-human epidermal growth factor receptor (HER) 2 antibody trastuzumab.26 CD3 co-targeting mAb-Fvs were constructed by fusing a humanized version (unpublished results) of the antibody OKT327 to the C-termini of hetero-Fc variants of trastuzumab and a humanized version (unpublished results) of an anti-HM1.24 antibody.28 Hetero-Fc selectivity was engineered using the HA-TF variant pair. In order to make the mAb-Fvs selective for their specified co-target antigen, we additionally incorporated a double variant G236R/L328R (referred to as FcKO) that ablates binding to FcγRs and complement. Linkers connecting the heavy chain to the C-terminal VH and VL domains were composites of IgG hinge sequences and glycine-serine repeats, and varying compositions and lengths were explored in preliminary constructs (data not shown). Final linkers for the three mAb-Fvs presented in this study are described in the Materials and Methods section.

Production and characterization of mAb-Fvs. mAb-Fv is made by co-expressing three distinct chains—a light chain and two different heavy chains. Heterodimerization of the two heavy chains is necessary to form a functional C-terminal Fv. mAb-Fvs and control antibodies were expressed in HEK293E cells and

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**Figure 1.** Engineering of the Fc/scFv-Fc system generates variant C3 domain pairs with enhanced heterodimerization. (A) Illustration of the Fc/scFv-Fc format. The C3 domain is shaded indicating where amino acid substitutions are made that promote Fc heterodimerization. (B) List of selected C3 domain pairs and introduced substitutions. (C) Virtual gel generated by the Agilent 2100 Bioanalyzer software allowing for visualization of the three dimeric species of the Fc/scFv-Fc system under non-reducing conditions. 1, molecular weight marker, 4.5 kDa; 2, system peak; 3, Fc monomer; 4, Fc homodimer; 5, Fc/scFv-Fc heterodimer; 6, scFv-Fc homodimer; 7, molecular weight marker, 240 kDa. (D) Summary of relative amounts by mass of the three dimeric species. Empty bar designates Fc homodimer; filled bar designates Fc/scFv-Fc heterodimer; cross-hatched bar designates scFv-Fc homodimer. Monomeric species were also detected but totaled less than 3%. (E) Electropherograms of Fc/scFv-Fc system for native IgG1 and HA-TF variant domain pair. Peaks are labeled as in (C). (F) Thermal denaturation of Fc homodimers with the indicated variant C3 domain pairs as measured by differential scanning calorimetry.
to calculated MWs of the light chain (~23 kDa) and two heavy chains (~65–67 kDa). Under non-reducing conditions a dominant main band at ~190 kDa was observed for each mAb-Fv, corresponding to the disulfide-linked tetramer (two heavy chains, each complexed with a light chain—calculated MW 179–181 kDa). The consistent results for the αCD16 and two αCD3 constructs indicate that the platform is fairly robust.

Heterodimer yield was not measured for the mAb-Fvs due to the approximately equal MWs of the three dimeric combinations; however, as observed for the Fc/scFv-Fc system, some amount of homodimer is likely present. To gain more insight into how the C-terminal Fv affects heterodimerization, we produced one-arm versions of the αHER2 x αCD16 mAb-Fv (Fig. S4).

For these mAb-Fvs, in which one of the heavy chains lacks V\text{H} and C\text{H1} domains, the three dimeric combinations have different MWs and can be distinguished electrophoretically. Under non-reducing conditions, a dominant (85%, 88% by mass) main band at ~140 kDa was observed for each one-arm mAb-Fv (two heavy chains, one of which is complexed with a light chain—calculated MW 131 kDa), corresponding to the desired heterodimeric trimer. These results suggest that the C-terminal Fv is not interfering with the heterodimerization promoted by the HA-TF variant Fc.

We analyzed mAb-Fvs using size-exclusion chromatography (SEC) to measure their hydrodynamic volume and assess the

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**Figure 2.** Structural models of the C\text{3} domains of lead variants promoting Fc heterodimerization. (A) S364H-Y349T heterodimer, (B) S364H-S364H homodimer, (C) Y349T-Y349T homodimer, (D) F405A-T394F heterodimer, (E) F405A-F405A homodimer, (F) T394F-T394F homodimer. A putative hydrogen bond between S364H and Y349T is indicated by a dashed line in (A). In (A–C), the C-terminus is oriented toward the reader; in (D–F), the C-terminus is oriented away from the reader.

**Figure 3.** Illustration of the mAb-Fv format (A) relative to a native IgG1 antibody (B). The additional monovalent Fv of the mAb-Fv is indicated with stripes. The variant C\text{3} domain of the mAb-Fv is shaded indicating amino acid substitutions made to promote Fc heterodimerization.
mAb-Fvs selectively co-engage CD16 and mediate potent antibody-dependent cell-mediated cytotoxicity. In order to confer selective CD16 binding, we constructed the αHER2 x αCD16 mAb-Fv in the context of a variant Fc containing (in addition to the HA-TF hetero-Fc variant) substitutions G236R and L328R that ablate binding to FcγRs and complement (referred to as FcKO). C-terminal fusion of the 3G8 Fv, which binds only CD16, thus builds FcγR selectivity into the mAb-Fv. We measured FcγR binding of mAb-Fv and comparator antibodies by surface plasmon resonance (SPR) using a protein A/antibody capture format with Fc receptor as analyte. The mAb-Fv bound human CD16 with higher affinity than native IgG1 trastuzumab (Fig. 5A and D). Fits of the binding data provided equilibrium dissociation constants ($K_D$s) of 160 nM for the mAb-Fv relative to 550 nM for native IgG1 (3.4-fold overall affinity enhancement, Fig. 5D), in contrast to the FcKO variant trastuzumab that does not bind. As a comparator, a trastuzumab antibody Fc-engineered for enhanced effector function (S239D/I332E, referred to as DE) had much higher CD16 affinity ($K_D$ = 4.9 nM), similar to results from previous work in reference 29 and 30. To compare with monomeric 3G8 parent antibody, we measured CD16 affinity of His6-tagged 3G8 anti-CD16 Fab by SPR using a Fab capture format with receptor as analyte. The affinity of the 3G8 Fab ($K_D$ = 7.5 nM) was stronger than the mAb-Fv by over 20-fold. The majority of the mAb-Fv affinity loss was due to reduced on-rate, and the off-rate was reduced by only 2.8-fold relative to the Fab. These results suggest that the compromised affinity of the mAb-Fv is due at least in part to oligomeric order of the samples (Fig. 4B). The main peak for all of the mAb-Fvs eluted at a time that, based on the MW standard (not shown), corresponded to the mAb-Fv tetramer. Larger species were observed in the chromatograms of the αCD3 mAb-Fvs. The lack of marked larger bands in denaturing non-reducing gels suggested they were not disulfide linked, and thus we attributed these species to noncovalent multimers or aggregates. However, the absence or much lower prominence of the aggregate peaks for the αCD16 mAb-Fv, as well as in mAb-Fvs engineered with other C-terminal Fvs (data not shown), implicate the biophysical properties of the C-terminal OKT3 Fv in their formation. This difference in behavior between αCD3 mAb-Fvs and other mAb-Fvs was reproducible across numerous expressions and protein preparations. These results suggest Fv optimization as an approach to improve the αCD3 mAb-Fv, and more generally as a valuable refinement step for the platform.

**Figure 4.** Characterization of mAb-Fv bispecifics. (A) Virtual gel summarizing electrophoresis of mAb-Fv bispecifics and αHER2 IgG1 control under both non-reducing and reducing conditions. (B) Size-exclusion chromatography of mAb-Fv bispecifics and αHER2 IgG1 control. 1, mAb-Fv dimer/aggregate; 2, mAb-Fv at expected 180 kDa peak; 3, mAb at expected 150 kDa peak.
The distal binding site of this receptor to both the FcγR binding site affected by the FcKO substitutions, the dimer interface affected by the HA-TF substitutions, and the 3G8 connection site at the CH3 C-terminus.

We tested the capacity of the αHER2 x αCD16 mAb-Fv to mediate cellular killing against HER2+ cells in a cell-based ADCC assay. Trastuzumab native IgG1 exhibited a moderate level of cytotoxicity against SK-BR-3 cells (EC$_{50}$ = 44 pM, maximal lysis = 23%) (Fig. 6). The mAb-Fv mediated a greater level of killing activity in terms of both potency (EC$_{50}$ = 6.1 pM, 7.2-fold) and maximal level of lysis (32%, 1.4-fold), and was comparable in potency to the DE Fc variant version. Control antibody with steric hindrance from tethering of the 3G8 Fv domain at both V$_{H}$ and V$_{L}$ N-termini.

In contrast to CD16 binding, both mAb-Fv and FcKO lacked binding to CD32a (Fig. 5B and D), as well as all of the other FcγRs (data not shown), highlighting the Fc receptor selectivity of the mAb-Fv relative to native IgG1 trastuzumab. The DE variant antibody showed much higher affinity to CD32a, again consistent with previous work in references 29 and 30.

Finally, mAb-Fv and all antibodies bound comparably to the neonatal Fc receptor FcRn (Fig. 5C and D), which plays an important role in maintaining the serum half-lives of antibodies. The uncompromised FcRn affinity is consistent with the distal binding site of this receptor to both the FcyR binding site affected by the FcKO substitutions, the dimer interface affected by the HA-TF substitutions, and the 3G8 connection site at the C$_{H3}$ C-terminus.

We tested the capacity of the αHER2 x αCD16 mAb-Fv to mediate cellular killing against HER2+ cells in a cell-based ADCC assay. Trastuzumab native IgG1 exhibited a moderate level of cytotoxicity against SK-BR-3 cells (EC$_{50}$ = 44 pM, maximal lysis = 23%) (Fig. 6). The mAb-Fv mediated a greater level of killing activity in terms of both potency (EC$_{50}$ = 6.1 pM, 7.2-fold) and maximal level of lysis (32%, 1.4-fold), and was comparable in potency to the DE Fc variant version. Control antibody with
no specificity for HER2 (αRSV) had no activity. The comparable activity of the mAb-Fv relative to DE and much greater activity relative to IgG1 were inconsistent with their CD16 affinities—
the CD16 affinity of the mAb-Fv was 34-fold lower than DE, but only 3.4-fold greater than IgG1. A possible explanation is that the more important parameter for effector function is not affinity but off-rate, which, as noted for the mAb-Fv, was only marginally compromised relative to its reduced on-rate. The off-rates of the mAb-Fv and DE variant mAb were improved by 7.8- and 27-fold relative to native IgG1, respectively (Fig. 5D). Other factors may include the particular epitope targeted by the 3G8 antibody, the distance and structure of the cell-to-cell complex, and potentially the selectivity of the mAb-Fv for CD16+ NK cells relative to other FcγR+ cells in the PBMC mixture. Additional studies are needed to understand the differences in mAb-Fv activity relative to the IgG1 and Fc-optimized antibodies.

**mAb-Fvs co-engaged CD3 and mediated T-cell dependent cytotoxicity.** We measured the affinity of the αHER2 x αCD3 mAb-Fv for T cells in a competitive binding experiment with labeled OKT3 antibody. The mAb-Fv bound CD3 with an IC_{50} of 38 nM (Fig. 7). In contrast to the αCD16 mAb-Fv results, the affinity was comparable to the humanized OKT3 parent Fab (IC_{50} = 44 nM).

We tested the capacity of the mAb-Fvs to mediate cytotoxic killing against tumor cells by both PBMCs and purified T cells. With PBMCs as effectors, both αHER2 x αCD3 and αHM1.24 x αCD3 mAb-Fvs mediated potent activity against HER2– Calu-3 lung cancer cells and HM1.24+ RPMI8266 myeloma cells, respectively (Fig. 8A and B). The potency of both αCD3 mAb-Fvs was inferior to the respective Fc-engineered (DE) antibodies. However, while the maximal lysis of the Fc-engineered version was greater for αHER2, the αHM1.24 x αCD3 mAb-Fv showed greater overall lysis compared with the Fc-engineered antibody. The mechanistic differences between the activities of the mAb-Fvs and Fc-engineered antibodies (T-cell-mediated vs. NK cell-mediated, respectively), preclude a simple comparison of the two activities.

Use of isolated T cells as effectors directly demonstrated the engineered CD3-mediated activity of the mAb-Fvs. Both αHER2 (Fig. 8C) and αHM1.24 (Fig. 8D) versions of the CD3-targeting mAb-Fv mediated potent cytotoxic activity by T cells, in contrast to both the native IgG1 and FcKO antibodies that showed none. Marginal activity was observed in the non-target mAb-Fv controls (αHM1.24 x αCD3 for the HER2+ cells and αHER2 x αCD3 for the HM1.24+ cells) relative to the IgG1 and FcKO antibodies. The possibility that this activity was real was supported by similar results from the αCD3 Fab, suggesting that non-specific engagement of CD3 can elicit some level of T-cell activation. Regardless, the strong T-cell-mediated tumor lysis by the mAb-Fvs relative to the native IgG1 antibody directly demonstrates the CD3-mediated activity of the mAb-Fvs, and overall illustrates the potential of the format for enabling powerful new functional activities.

**Discussion**

Natural biological activation of immune receptors is often accomplished by cross-linking, mediated, for example, by antibody/antigen immune complexes or effector cell/target cell synapses. This principle is a consideration for engineering new binding sites into therapeutic antibodies because cross-linking in the absence of target can cause nonspecific activation. The clinical toxicity that results from nonspecific crosslinking of CD3 by antibodies\(^{15,16}\) is emblematic of this issue of valence. Hence for some bispecific targets the preferred mode of binding may be monovalent such that activation occurs only upon encountering
identified by structural calculations, it seems to be stabilized by a putative hydrogen bond between the imidazole ring of S364H and the hydroxyl group of Y349T. Variant A-F, identified from examination of the homologous CH1/CL heterodimer, is similar to a previously published knob-into-hole variant (F405A-T394W). The behavior of the mAb-Fv was suboptimal in three regards—the lack of 100% heterodimer yield, the presence of noncovalent multimers or aggregates for the αCD3 mAb-Fvs, and the reduced affinity of the αCD16 C-terminal Fv relative to parent Fab. In the Fc/scFv-Fc system, the heterodimer yield of our HA-TF variant pair did not reach 100% and was dependent on the relative expression levels of the two heavy chains. Heterodimer yield has not yet been quantified for the three mAb-Fvs presented, but some unwanted homodimer likely remains. Further studies are needed to clarify the optimal strategy for its removal. The larger peaks in the αCD3 mAb-Fv size exclusion chromatograms were not observed for mAb-Fvs with αCD16 or other C-terminal Fvs (not shown). These results imply that the C-terminal Fv plays a role in mAb-Fv fidelity, and thus suggest a potential engineering solution via Fv optimization. Similar to results observed for other bispecifics with N-terminally constrained Fv regions, the αCD16 C-terminal Fv has compromised affinity for its opsonized, and thus highly avid, primary target cell. Although fragment-based antibody bispecifics are capable of binding antigen monovalently, they typically suffer from rapid clearance in vivo and deficiencies in production and stability. While bispecific formats based on full-length antibodies are more optimal because they benefit from the stability, size and favorable receptor-binding properties of the Fc region, the homodimeric nature of the heavy chain imposes bivalency on full-length bispecific antibodies. The mAb-Fv was designed as an architectural solution to this problem.

While the mAb-Fv doesn’t require a variant Fc, we reasoned that promoting heterodimerization was the most productive approach. Structure- and sequence-guided methods have previously been used to successfully engineer heterodimeric Fc regions using strategies such as knobs-into-holes,22 electrostatic steering,23 and IgG/IgA strand-exchange.31 Our design strategy coupled structural calculations and sequence information to narrow the diversity to a size (41 variant pairs) amenable to screening in linear throughput. Rational combination of favorable substitutions resulted in our lead variant pair HA-TF, a combination of S364H-Y349T (H-T) and F405A-T394F (A-F) that shifted the unbiased 50% distribution to 83% heterodimer. Variant H-T, identified by structural calculations, seems to be stabilized by a putative hydrogen bond between the imidazole ring of 364H and the hydroxyl group of 349T. Variant A-F, identified from examination of the homologous C_1α/C_1β heterodimer, is similar to a previously published knob-into-hole variant (F405A-T394W).24 The behavior of the mAb-Fv was suboptimal in three regards—the lack of 100% heterodimer yield, the presence of noncovalent multimers or aggregates for the αCD3 mAb-Fvs, and the reduced affinity of the αCD16 C-terminal Fv relative to parent Fab. In the Fc/scFv-Fc system, the heterodimer yield of our HA-TF variant pair did not reach 100% and was dependent on the relative expression levels of the two heavy chains. Heterodimer yield has not yet been quantified for the three mAb-Fvs presented, but some unwanted homodimer likely remains. Further studies are needed to clarify the optimal strategy for its removal. The larger peaks in the αCD3 mAb-Fv size exclusion chromatograms were not observed for mAb-Fvs with αCD16 or other C-terminal Fvs (not shown). These results imply that the C-terminal Fv plays a role in mAb-Fv fidelity, and thus suggest a potential engineering solution via Fv optimization. Similar to results observed for other bispecifics with N-terminally constrained Fv regions, the αCD16 C-terminal Fv has compromised affinity for its...
antigen. This is likely due to steric hindrance, a hypothesis that is supported by the reduced on-rate, but marginally affected off-rate, of the αCD16 mAb-Fv relative to the 3G8 Fab. Overall, the observed deficiencies may be a result of using two antibodies from the literature, 3G8 and OKT3, as our initial proof of concept, a choice based simply on availability. Screening at the discovery stage or in optimization libraries for antibodies that have higher starting affinity, greater pairing stability, or more tolerance to linkage at their N-termini is an obvious next step for refinement of the mAb-Fv format.

In line with the previous discussion, it would seem that a mAb-Fab format, essentially the mAb-Fv plus the C\textsubscript{H}1 and C\textsubscript{L} constant chains at the C-terminus, would be more favorable. We had initiated this work with the mAb-Fab as our original architecture, but the expressed mAbs species were complex and protein behavior was more problematic than the mAb-Fv (data not shown). We suspect that this result was due to diverse irreversible pairing of the C-terminal Fab chains with the free light chain, coupled with the complex role of C\textsubscript{H}1/C\textsubscript{L} assembly in export.\textsuperscript{33}

The potent and selective cytotoxic activity of both the αCD16 and αCD3 mAb-Fvs confirmed the formation of the C-terminal Fv, and directly illustrated the value of the format. The T-cell-mediated activity in particular is appealing. Previous studies have demonstrated CD3-mediated cytotoxic killing by bispecific antibody fragments,\textsuperscript{34,35} and the impressive clinical activity of these biotherapeutics\textsuperscript{6} has undoubtedly motivated revived efforts at bispecifics. Our results indicate that it is possible to build these redirected activities into a format that is more favorably like a full-length antibody, particularly with respect to serum half-life.

Since the mid-1980s,\textsuperscript{6} bispecific antibodies have been explored for two principal applications. One is the consolidation of two drugs into one, examples of which include IL-12(IL-18,\textsuperscript{3} IL-1α/IL-1β,\textsuperscript{4} HER2/VEGF,\textsuperscript{3} and EGFR/doxorubicin.\textsuperscript{30} While such uses may arguably simplify drug development or clinical application, they ultimately reduce dosing options. Consequently, an accurate understanding of the relationship between affinities for the two targets and therapeutic window must be known at the discovery stage, a case that rarely exists. Our interest in bispecific targeting is for its other use, namely the enablement of novel activities that are attainable only via engineered co-engagement. Historically such application has typically involved targeting an effector cell to a tumor, e.g., by co-engaging a cancer antigen and an immune receptor such as CD16, CD64 or CD3. Yet, as demonstrated by recent work with co-engagement of TNF receptor family members\textsuperscript{10} and CD32b/B-cell receptor complexes,\textsuperscript{38,39} powerful de novo immunomodulatory activities can be engineered for both oncology and inflammatory/autoimmune applications. Given that the majority of such co-engagement goals involve immune receptors that have evolved to fire upon cross-linking, the capacity to co-engage distinct target antigens with different valencies is a valuable new capability for formatting bispecifics for optimal therapeutic use.

Materials and Methods

Protein design. Fc variants that promote heterodimerization were designed using a combination of structure- and sequence-based approaches. Computational structure-based methods\textsuperscript{21} were used to evaluate possible amino acid substitutions in the C\textsubscript{H}3 region for their ability to stabilize Fc heterodimers and destabilize Fc homodimers. Sixteen pairs of inter-chain positions were selected for evaluation based on their proximity across the C\textsubscript{H}3 homodimer interface.\textsuperscript{40} Energies for 19\textsuperscript{2} = 361 amino acid pairs (cysteine was excluded) were calculated in the context of the heterodimer (both variant amino acids) and the two homodimers (variant amino acid at one position and the native amino acid at the other) and were normalized against the calculated energy of the native amino acid pair. The preference for the heterodimeric species was calculated according to the following equation:

Heterodimer Preference = E(heterodimer) - average [E(homodimer 1), E(homodimer 2)]

An example of this analysis for positions 349 and 364 is illustrated in Figure S5. Design was further guided by sequence information from naturally heterodimeric immunoglobulin (Ig) domains. A structural alignment of the human C\textsubscript{H}3 homodimer, human C\textsubscript{H}1/C\textsubscript{H}κ heterodimer and human C\textsubscript{H}1/C\textsubscript{H}λ heterodimer was generated using the web-based tool MultiProt.\textsuperscript{41} The resulting sequence alignment was examined for inter-chain pairs with contrasting biophysical properties.

Reagents. Genes encoding human CD16 (FcγRIIIa) and CD32a were obtained from the Mammalian Gene Collection (American Type Culture Collection). Extracellular domains were subcloned into the vector pcDNA3.1Zeо (Invitrogen) with a C-terminal His\textsubscript{6} tag. DNA was transfected into HEK293T cells and receptor proteins were purified using nickel affinity chromatography (Qiagen, Valencia, CA). Human neonatal Fc receptor protein (FcRn) α chain and β-2-microglobulin genes were obtained from OriGene Technologies (Rockville, MD). Extracellular domains were subcloned into the vector pcDNA3.1Zeо (Invitrogen). DNA was cotransfected into HEK293T cells and receptor protein was purified by affinity chromatography using an antibody variant with high affinity for FcRn.\textsuperscript{42}

Construction, expression and purification. The Fc/scFv-Fc system was constructed using an scFv derived from murine anti-CD3 (αCD3) antibody OKT3.\textsuperscript{37} DNA encoding the OKT3 scFv was generated by gene synthesis (Integrated DNA Technologies, San Diego, CA) and was subcloned using standard molecular biology techniques into the expression vector pTT5\textsuperscript{20} encoding a native human heavy IgG1 Fc region. Substitutions in the Fc domain were introduced using site-directed mutagenesis (QuikChange, Stratagene, Cedar Creek, TX). Positions are numbered according to the EU index.

The αHER2 x αCD16 mAb-Fv was constructed in the pTT5 vector\textsuperscript{20} using a combination of gene synthesis (Blue Heron Biotechnology, Bothell, WA), subcloning and site-directed mutagenesis (QuikChange, Stratagene, Cedar Creek, TX). The linker used to connect the C\textsubscript{H}3 domain to the anti-CD16 V\textsubscript{H} or V\textsubscript{L} was “SSD KTH TSP PSP GGG GSG GGG SGG GGS GGG G” and...
was included in the subcloning. The αHER2 variable region was that of trastuzumab26 and the αCD16 variable region was murine antibody 3G8,23 both of which were made by gene synthesis. The Fc-knockout (FcKO) substitutions G236R/L328R were made in the αHER2 x αCD16 mAb-Fv using site-directed mutagenesis (QuikChange, Stratagene, Cedar Creek, TX). αHER2 x αCD3 and αHM1.24 x αCD3 mAb-FvVs were derived from the αHER2 x αCD16 mAb-Fv construct by additional gene synthesis and subcloning. For these constructs the linker used to connect the Cγ3 domain to the αCD3 Vγ or Vα was “SSD KTH TSP PSP SG” and was included in the αCD3 gene synthesis. The αCD3 variable region was a humanized version (unpublished results) of the murine antibody OKT3.27 The αHM1.24 variable region was a humanized version (manuscript in preparation) of the antibody HM1.24.28 mAb-Fv constructs incorporated detection/purification tags on the C-termini, including His6-tags and FLAG-tags, although these tags were not used in these experiments.

Effecter function-enhanced (S239D/I332E, referred to as DE) and -knockout (G236R/L328R, FcKO) versions of αHER2 and αHM1.24 IgG antibodies were built from previous constructs29,30 via subcloning. 3G8 anti-CD16 and humanized OKT3 anti-CD3 FabGs were constructed using gene synthesis (Blue Heron) with a His6-tag on the heavy chain C-terminus. DNA encoding all proteins (Fc, scFv-Fc, mAb-FvVs and control IgGs and Fabs) were cotransfected into HEK293E cells20 for expression and resulting proteins were purified from the supernatant. For these constructs the linker used to connect the Cγ3 domain to the αCD3 Vγ or Vα was “SSD KTH TSP PSP SG” and was included in the αCD3 gene synthesis. The αCD3 variable region was a humanized version (unpublished results) of the murine antibody OKT3.27 The αHM1.24 variable region was a humanized version (manuscript in preparation) of the antibody HM1.24.28 mAb-Fv constructs incorporated detection/purification tags on the C-termini, including His6-tags and FLAG-tags, although these tags were not used in these experiments.

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Protein analysis. Electrophoresis was performed using an Agilent 2100 Bioanalyzer microfluidics-based platform (Agilent Technologies, Santa Clara, CA). Proteins were diluted to 1 mg/mL in PBS and run on Agilent Protein 230 chips following the manufacturer’s protocol. Proteins were run under reducing and non-reducing conditions in parallel. Protein fractions were quantified by relative absorbance using the instrument software. Size-exclusion chromatography was performed using an Agilent 1200 High-performance liquid chromatography system. Samples were injected onto a Superdex 200 10/300 GL column (GE Healthcare) at 0.5 mL/min using PBS, pH 7.4 as the mobile phase. A MW standard (Bio-Rad Laboratories, Hercules, CA) was run (data not shown). Thermal denaturation experiments were performed using a MicroCal VP-DSC MicroCalorimeter (GE Healthcare). Stability experiments were performed in an Fc/Fc system to avoid complication of the data from the unfolding of the αCD3 scFv. Variant Fc constructs were transfected alone or cotransfected to generate desired homodimeric or heterodimeric species for analysis. Proteins were diluted to 0.5 mg/mL in PBS, degassed and heated from 25 to 100°C using a heating rate of 1.5°C/min. The observed melting profiles were baseline-corrected and normalized using the instrument software.

Determination of binding affinities. Binding to Fc receptors was measured by surface plasmon resonance (SPR) using a Biacore 3000 instrument (GE Healthcare). FcγR affinity of αHER2 x αCD16 mAb-Fv and control/comparator full-length antibodies was determined using a protein A chip/antibody capture format with receptor as analyte as described previously in reference 30. CD16 affinity of His6-tagged 3G8 αCD16 Fab was measured using an anti-human Fab chip/Fab capture format with CD16 receptor as analyte. FcRn binding by αHER2 x αCD16 mAb-Fv and antibodies was measured using a HER2 chip/antibody capture format with FcRn receptor as analyte similar to as described previously in reference 42. For these experiments anti-Fab and HER2 chips were generated by conjugating recombinant goat anti-human Fab F(ab’)2 (Jackson ImmunoResearch Lab, West Grove, PA) or HER2-Fc (R&D Systems) to a CM5 chip (GE Healthcare) using standard primary amine coupling. For all Biacore experiments, data were processed by zeroing time and response before the injection of receptor and by subtracting appropriate nonspecific signals (response of reference channel and injection of running buffer). Equilibrium constants were calculated by global fitting of binding data with a conformational change model (in the case of CD16 and FcRn) or a Langmuir 1:1 binding model (CD32a) using BLAevaluation software (GE Healthcare).

Binding of αHER2 x αCD3 mAb-Fv and controls to CD3 was measured on T cells in a competition experiment with labeled OKT3 antibody, mAb-Fv, comparator humanized OKT3 Fab, or control antibodies at various concentrations were preincubated with ~1 million PBMCs on ice for 1 h. FITC-labeled OKT3 (BioLegend, San Diego, CA) at a final concentration of 25 ng/ml plus APC-labeled αCD16 and αCD20 antibodies (BD Biosciences, San Diego, CA) were added and incubated for an additional 45 min in 3% FBS/PBS. Samples were washed twice, fixed with 1% paraformaldehyde/PBS and analyzed using a FACSCanto II flow cytometer (BD Biosciences). T cells were identified as CD16 CD20+ live lymphocytes; approximately 3,000 events were captured per sample. Binding of FITC-OKT3 to T cells was quantified by mean fluorescence intensity (MFI) values, which were then plotted against the log of the concentrations. Data was fitted with a four-parameter sigmoidal dose-response curve using Prism 5 (GraphPad Software) and the relative affinities were determined as IC50 values of each curve.

Cytotoxicity assays. CD16- and CD3-mediated cellular killing by PBMCs or purified T cells was determined by lactate dehydrogenase (LDH) release. Human peripheral blood mononuclear cells (PBMCs) were purified from leukapheresis of anonymous healthy volunteers (HemaCare, Van Nuys, CA) using Ficoll-Paque Plus density gradients (Amersham Biosciences, Newark, NJ). Primary human T cells were purified from PBMCs by negative selection using a T Cell Enrichment Kit (StemCell Technologies, Vancouver, British Columbia). SK-BR-3, Calu-3 and RPMI8266 cell lines were obtained from American Type Culture Collection. Tumor target cells were seeded into 96-well plates at 10,000 cells/well and opsonized with antibodies in triplicate at the indicated final concentrations. For the anti-CD16 ADCC assay, PBMCs were added at 50:1 effector to target ratio (E:T) and plates were incubated at 37°C for 4 h. For the anti-CD3 killing assay with PBMCs as effectors, E:T was 50:1 and incubation time was 24 h. For the anti-CD3 killing assay with
T cells as effectors, E:T was 20:1 and incubation time was 24 h. Triton X-100 and effector cells alone were run as controls for calculation of % lysis. Cells were then incubated with LDH reaction mixture (CytoTox-ONE, Promega) for 10 min and fluorescence was measured using a Wallac Victor2 fluorometer (Perkin-Elmer). Data was normalized to maximal (Triton) and minimal (effectors alone) lysis and fit to a sigmoidal dose-response using Prism 5 (GraphPad Software).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at: www.landesbioscience.com/journals/mabs/articles/18123

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