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Elucidating heavy/light chain pairing preferences to facilitate the assembly of bispecific IgG in single cells

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ABSTRACT

Multiple strategies have been developed to facilitate the efficient production of bispecific IgG (BsIgG) in single host cells. For example, we previously demonstrated near quantitative (≥90%) formation of BsIgG of different species and isotypes by combining 'knob-into-hole' mutations for heavy chain heterodimerization with engineered antigen-binding fragments (Fabs) for preferential cognate heavy/light chain pairing. Surprisingly, in this study we found high yield (>65%) of BsIgG$_1$ *without* Fab engineering to be a common occurrence, i.e., observed for 33 of the 99 different antibody pairs evaluated. Installing charge mutations at both C$_H$1/C$_L$ interfaces was sufficient for near quantitative yield (>90%) of BsIgG$_1$ for most (9 of 11) antibody pairs tested with this inherent cognate chain pairing preference. Mechanistically, we demonstrate that a strong cognate pairing preference in one Fab arm can be sufficient for high BsIgG$_1$ yield. These observed chain pairing preferences are apparently driven by variable domain sequences and can result from a few specific residues in the complementarity-determining region (CDR) L3 and H3. Transfer of these CDR residues into other antibodies increased BsIgG$_1$ yield in most cases. Mutational analysis revealed that the disulfide bond between heavy and light chains did *not* affect the yield of BsIgG$_1$. This study provides some mechanistic understanding of factors contributing to antibody heavy/light chain pairing preference and subsequently contributes to the efficient production of BsIgG in single host cells.

Introduction

Bispecific antibodies (BsAb) can target two epitopes on the same or different antigens. BsAb can support novel mechanisms of action that are not available to monospecific antibodies, and thereby offer a promising approach to next-generation antibody drugs.[1,2] Novel mechanisms of action for bispecifics are exemplified by the two currently marketed BsAb, namely, blinatumomab (anti-CD19/CD3) and emicizumab (anti-factor IXa/factor X).[2] Blinatumomab directs T cells to kill tumor cells expressing CD19 and is approved for the treatment of B cell precursor acute lymphoblastic leukemia.[3] Emicizumab mimics the function of factor VIII in promoting the formation of a factor IXa/factor X complex and is approved for the treatment of hemophilia A.[4,5] Over 100 different BsAb formats have been described, including at least 10 different formats that have reached clinical trials.[1]

BsIgG is a widely used tetrameric BsAb format commonly consisting of two different light chains (LC) and two different heavy chains (HC). Production of BsIgG in single host cells can be difficult due to low yield of the desired bispecific and the formidable purification challenge of removing closely related IgG contaminants. These challenges result from co-expression of the four component chains (two LC and two HC), which may assemble into nine different mispaired IgG species in addition to the correctly paired BsIgG.[6] Undesired chain mispairing events associated with BsIgG production in single cells include formation of HC homodimers and non-cognate HC/LC pairs.[1,7]

Several different strategies have been developed to overcome or avoid chain mispairing to allow efficient production of BsIgG for drug development.[1,7] For example, efficient assembly of HC heterodimers with minimal homodimers was first achieved in the 1990s using 'knob-into-hole' (KIH) mutations.[8–10] Subsequently, several other methods for efficient HC heterodimerization were developed.[1] Alternatively, the HC pairing problem has been entirely avoided by using a common HC, as demonstrated with κλ-bodies.[11]

LC mispairing was initially circumvented using a common LC,[9] and more recently by expression of engineered half-IgG in separate host cells followed by *in vitro* assembly into IgG.[12–14] Efficient assembly of cognate HC/LC pairs has been accomplished by domain swapping with the CrossMab format.[15] Subsequently, orthogonal antigen-binding fragment (Fab) interfaces were developed[16] and further improved[17] to favor cognate over non-cognate HC/LC pairing. Additional successful strategies developed for engineering Fab domain interfaces include replacement of a native inter-chain disulfide bond with an engineered one[18,19]

and installation of charge pairs either alone[20,21] or in combination with KIH mutations.[21]

Previously we transiently expressed BsIgG in single mammalian cells (Expi293F) by co-transfection of DNAs encoding the constituent HC and LC.[21,22] The resultant IgG species was purified from the cell culture supernatant by protein A chromatography. The BsIgG yield, defined here as the percentage of correctly assembled BsIgG in the purified pool of different IgG species, was estimated by high resolution and accurate mass liquid chromatography-mass spectrometry (LCMS) using an extended mass range (EMR) Orbitrap mass spectrometer.[21,22] The HC in the BsIgG included KIH mutations for preferential HC heterodimerization, and the Fab was engineered to favor cognate over non-cognate HC/LC pairing.[21] Control BsIgG were constructed with KIH HC mutations but *without* Fab engineering. For some control BsIgG such as anti-HER2/CD3 and anti-VEGFA/ANG2, the yield of BsIgG was 22–24%,[21] consistent with random pairing of HC and LC as previously observed by others.[23] In contrast, high BsIgG yield, arbitrarily defined here as >65%, was observed for other control bispecifics such as anti-EGFR/MET and anti-IL-4/IL-13 BsIgG, consistent with a cognate HC/LC pairing preference.[21] Examples of elevated BsIgG yield *without* Fab mutations have also been observed by others.[16,24]

Prior to this study, the prevalence of cognate HC/LC pairing preferences and the molecular basis for this phenomenon were only poorly understood. Here, high throughput production and high-resolution LCMS analysis[21,22] were utilized to survey 99 different antibody pairs with KIH HC, but *without* Fab mutations, for the yield of $BsIgG_1$. One-third of antibody pairs showed high (>65%) $BsIgG_1$ yield, consistent with a strong inherent cognate HC/LC chain pairing preference. Installation of previously identified charge mutations at the two C_H1/C_L domain interfaces[21] for such antibody pairs was used to enhance the production of $BsIgG_1$. Next, we investigated whether a cognate chain pairing preference in one or both arms was needed for high yield of $BsIgG_1$. Mutational analysis was used to identify specific residues in CDR L3 and H3 contributing to high $BsIgG_1$ yield. The CDR L3 and H3 and specific residues identified were then inserted into other available antibodies that show random HC/LC chain pairing to determine their effect upon $BsIgG_1$ yield. Finally, mutational analysis was used to investigate the effect of the interchain disulfide bond upon the yield of $BsIgG_1$.

Results

High yield of $BsIgG_1$ without Fab mutations is a common phenomenon that depends on the constituent antibody pairs

Previously, we observed high yield of BsIgG (>65%) with KIH HC but *without* Fab arm mutations for two bispecifics, namely, anti-EGFR/MET and anti-IL-13/IL-4.[21] Here we investigated the strength and frequency of occurrence of cognate HC/LC pairing preference. A large panel of antibody pairs (n = 99) was used to

generate bispecific IgGs. For simplicity, all bispecifics in this study were constructed with human IgG_1 HC constant domains. Six antibodies binding to either IL-13, IL-4, MET, EGFR, HER2 or CD3[21] were used to construct a matrix of all 15 possible $BsIgG_1$. Next, these six antibodies were permuted with 14 additional antibodies that were mainly κ LC isotype with three λ LC isotype (anti-DR5, anti-$\alpha_5\beta_1$, anti-RSPO2) (Table S1). All but three of the antibodies used in this study were humanized; the 3 exceptions were fully human antibodies (anti-CD33, anti-PDGF-C, anti-FluB). Antibody pairs were co-expressed at optimized chain ratios, and the yield of $BsIgG_1$ was determined with an improved version of our previously described method.[21,22] Briefly, following co-expression of antibody pairs and protein A chromatography, the purified IgG_1 pools were further purified by size exclusion chromatography (SEC) to remove any small quantities of aggregates and half IgG_1 present prior to quantitation by high-resolution LCMS. The yield of correctly assembled $BsIgG_1$ in isobaric mixtures that also contained LC-scrambled IgG_1 was estimated using a previously developed algebraic formula.[22]

The yield of $BsIgG_1$ for the 99 unique antibody pairs varied over a very wide range: 22–95% (Table 1). Strikingly, non-random HC/LC pairing (>30% yield of $BsIgG_1$) was observed for the majority (>80%) of antibody pairs, with high (>65%) and intermediate (30–65%) yield of $BsIgG_1$ seen for 33 and 48 antibody pairs, respectively. Near quantitative (>90%) formation of $BsIgG_1$ was measured for two antibody pairs (anti-MET/DR5 and anti-IL-13/DR5). High-resolution LCMS data for representative examples of low (anti-LGR5/IL-4, Figure 1(a,d)) intermediate (anti-SIRPα/IL-4, Figure 1(b,e)) and high (anti-MET/DR5, Figure 1(c,f)) yield of $BsIgG_1$ are shown. The $BsIgG_1$ yield for each antibody studied varied over a wide range depending upon its partner antibody. For example, the $BsIgG_1$ yield for the anti-MET antibody varied from as little as ~21% when paired with anti-IL-33 to as much as ~95% when paired with anti-DR5 (Table 1). To investigate any influence of 'knob' and 'hole' mutations on the cognate HC/LC pairing preference, $BsIgG_1$ were produced with the HC containing the 'knob' mutation in mAb1 and 'hole' mutations in mAb2 or *vice versa* (Table 1). The yield of $BsIgG_1$ was minimally influenced by which HC contained the 'knob' and 'hole' mutations in all cases (n = 15) tested (Table 1). The recovery of IgG species from 30 mL cultures by protein A chromatography varied over ~5-fold (1.5 to 8.0 mg, Figure S1).

Enhanced yield of $BsIgG_1$ using C_H1/C_L interface charge mutations for antibody pairs with a cognate HC/LC chain pairing preference

Previously, we used a combination of mutations at all four domain/domain interfaces (i.e., both V_H/V_L and both C_H1/C_L) in conjunction with KIH HC mutations for near quantitative assembly of BsIgG of different isotypes in single mammalian host cells.[21] Here, we readily identified antibody pairs that give a high yield of $BsIgG_1$ *without* any Fab mutations (Table 1). These high yields of $BsIgG_1$ are presumably due to differences in variable domain sequences rather than constant domains (IgG_1 C_H1 and κ

Table 1. Survey of antibody pairs to investigate BsIgG$_1$ yields *without* Fab mutations but with KIH HC mutations for HC heterodimerization. Different combinations of IgG$_1$ HC and LC pairs were co-transfected into Expi293F cells and the yield of BsIgG$_1$ determined by high-resolution LCMS following protein A chromatography and SEC. The yield of correctly assembled BsIgG$_1$ in isobaric mixtures that also contained LC-scrambled IgG$_1$ (see Figure 1 for representative examples) was estimated using a previously developed algebraic formula.[22] Data shown are the yield of BsIgG$_1$ from optimized LC DNA ratios. BsIgG$_1$ yields >65% are indicated in bold. The HC of mAb-1 contained the 'hole' mutations (T366S:S368A: Y407V)[8] and the HC for mAb-2 contained a 'knob' mutation (T366W).[8] NA, not applicable: monospecific antibodies.

mAb-2	mAb-1					
	IL-13	MET	EGFR	CD3	IL-4	HER2
IL-13	NA	**87.6**	**87.0**	**75.2**	**70.3**	**66.6**
MET	**86.6**	NA	**72.3**	60.7	53.1	59.9
EGFR	**86.3**	**72.4**	NA	23.9	45.4	22.0
CD3	**75.5**	54.8	32.5	NA	25.0	22.7
IL-4	**68.7**	58.0	44.1	26.9	NA	22.6
HER2	64.6	**65.4**	21.6	24.1	25.0	NA
DR5	**90.4**	**95.1**	53.3	53.4	53.8	34.7
FluB	**87.7**	**69.5**	52.3	32.0	60.8	**72.7**
RSPO3	**84.7**	58.6	**82.1**	40.6	26.0	22.0
Factor D 25D7 v1	**83.6**	**73.1**	**69.3**	**83.1**	35.5	**68.7**
RSPO2	**83.5**	51.1	**78.5**	38.7	22.3	**71.3**
IL-1β	**74.2**	63.5	**80.4**	**77.8**	63.7	**65.9**
GFRα1	**73.9**	40.6	**77.5**	**79.6**	33.5	**68.0**
PDGF-C	61.2	**71.0**	54.6	56.0	34.2	24.3
CD33	49.8	58.8	49.6	36.4	56.5	51.5
α$_5$β$_1$	45.9	62.2	31.0	41.4	48.4	**72.6**
IL-33	45.6	21.4	30.9	20.4	42.4	46.6
SIRPα	41.7	31.0	22.6	60.6	47.9	31.8
Factor D 20D12 v1	23.5	29.8	58.0	36.0	22.6	**69.6**
LGR5	21.7	56.2	53.8	23.6	22.8	22.1

100.0   75.0   50.0   25.0   0

BsIgG$_1$ yield (%)

C$_L$) that were identical in most cases. We hypothesized that for such antibody pairs mutations at the two C$_H$1/C$_L$ interfaces alone might be sufficient to enhance the bispecific yield close to 100%. Eleven different antibody pairs were selected, and the yield of BsIgG$_1$ was compared in the presence or absence of previously reported C$_H$1/C$_L$ domain interface charge mutations.[21] Specifically, the 'knob' arms were engineered with C$_L$ V133E and C$_H$1 S183K mutations and the 'hole' arm with C$_L$ V133K and C$_H$1 S183E mutations.[21] The charge mutations at the two C$_H$1/C$_L$ interfaces increased the BsIgG$_1$ yield for all antibody pairs by ~12–34% to ≥90% BsIgG$_1$ yield in the majority (9/11) of cases (Figure 2). The charge mutations at the two C$_H$1/C$_L$ interfaces, however, have little to no impact on antigen binding (Table S2).

Cognate heavy/light chain pairing preference in one arm of a bispecific IgG can be sufficient for high bispecific yield

The mechanistic basis for high yield of BsIgG$_1$ observed for some antibody pairs was investigated. Two antibody pairs, namely anti-EGFR/MET and anti-IL-13/IL-4, were selected for this study based on their high yield of BsIgG$_1$ without Fab mutations (Table 1).[21] *A priori*, either one or both Fab may exhibit a cognate HC/LC pairing preference contributing to the high yield of BsIgG$_1$. Three chain co-expression experiments were undertaken to distinguish between these possibilities. A single HC (HC1) with either 'knob' or 'hole' mutations was transiently co-expressed in Expi293F cells with its cognate LC (LC1) and a competing non-cognate LC (LC2) (Figure 3(a)). The resultant half IgG species were purified from the corresponding cell culture supernatant by protein A affinity chromatography, and the extent of cognate and non-cognate HC/LC pairing was assessed by high-resolution LCMS[21] (Figure 3(b)).

The anti-MET HC shows a strong preference for its cognate LC (~71%) over the non-cognate anti-EGFR LC, whereas the anti-EGFR HC shows only a slight preference for its cognate LC (~56%) over the non-cognate anti-MET LC (Figure 3(b)). The anti-IL-13 HC shows a strong preference for its cognate LC (81%) over the non-cognate anti-IL-4 LC, whereas the anti-IL-4 HC shows no preference (49%) for its cognate LC (Figure 3(b)). These data are consistent with the notion that the high BsIgG$_1$ yield for anti-EGFR/MET results from the strong and weak cognate HC/LC pairing preference for the anti-MET and anti-EGFR antibodies, respectively. In contrast, the high BsIgG$_1$ yield for anti-IL-13/IL-4 apparently
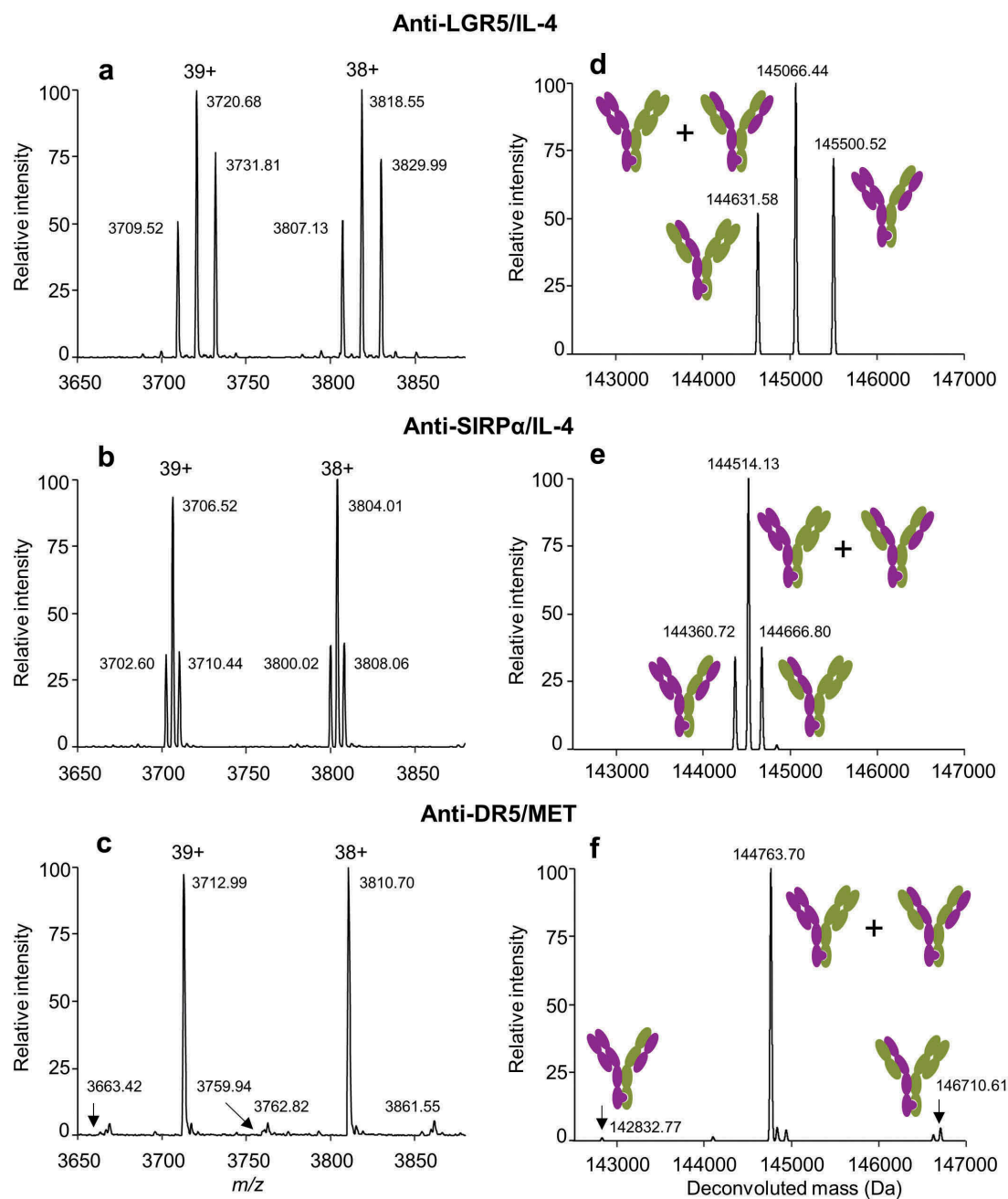
Figure 1. High-resolution LCMS analysis profiles highlighting representative examples from Table 1 for (a and d) low (<30%), (b and e) intermediate (30–65%) or (c and f) high (>65%) yield of BsIgG$_1$. Corresponding antibody pairs were transiently co-transfected into Expi293F cells. The IgG$_1$ species were purified by protein A chromatography and SEC before quantification of the BsIgG$_1$ yield by high-resolution LCMS.[21,22] (a-c) Data shown are mass envelopes for charge states 38+ and 39+ along with (d-f) corresponding deconvoluted data. Also shown are cartoons representing the different IgG$_1$ species present. See Table 1 for additional experimental details.

reflects a strong cognate HC/LC pairing preference for the anti-IL-13 antibody alone. Thus, a cognate HC/LC pairing preference in one or both arms can apparently be sufficient for high yield of BsIgG$_1$ in a single cell without the need for Fab mutations.

Anti-HER2/CD3 was selected as a control for this study based on its low yield of BsIgG$_1$ (Table 1).[21] The anti-HER2 HC shows no pairing preference for its cognate LC over the non-cognate anti-CD3 LC. Similarly, the anti-CD3 HC shows no pairing preference for its cognate LC over the non-cognate anti-HER2

LC (Figure 3(b)). However, in the absence of competition, HC can assemble efficiently with a cognate as well as with a non-cognate LC, as judged by all different matched and mismatched HC/LC pairs tested (Table S3).

CDR L3 and H3 of the anti-MET antibody contribute to high bispecific yield of anti-EGFR/MET BsIgG$_1$

We investigated the sequence determinants in the anti-MET antibody contributing to high bispecific yield of the anti-EGFR/MET
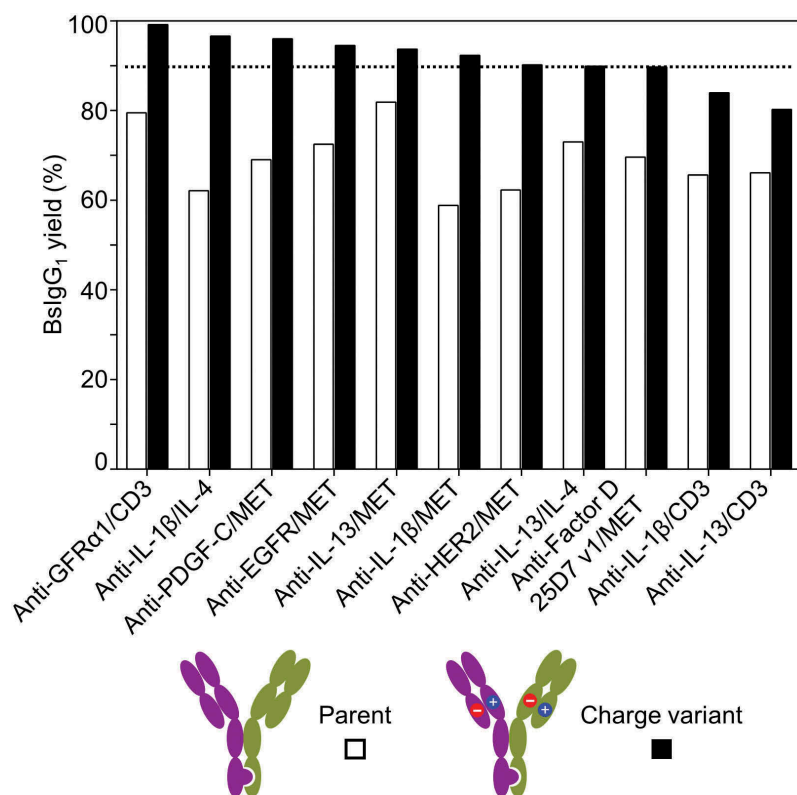
Figure 2. Incorporation of C_H1/C_L charge pair variants increases $BsIgG_1$ yields for antibody pairs that have a strong intrinsic HC/LC pairing preference. For the charge pair variants, the first listed antibody contains the C_L V133E and C_H1 S183K mutations and the second listed antibody contains the C_L V133K and C_H1 S183E mutations.[21] See Table 1 for additional experimental details. 90% yield of $BsIgG_1$ is indicated by a dotted horizontal line.
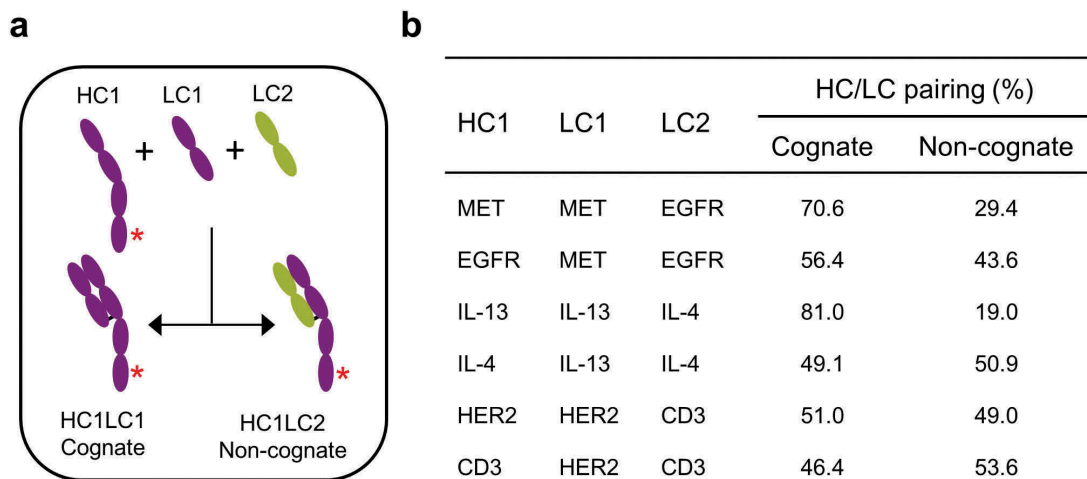

Figure 3. Investigation of cognate chain pairing preferences by co-expression of three chains. (a) Co-expression of an antibody heavy chain (HC1) with its cognate light chain (LC1) and a non-cognate light chain (LC2) in Expi293F cells. (b) Quantification of antibody cognate chain preferences following co-expression. The IgG_1 and half IgG_1 species were purified from the cell culture supernatant by protein A chromatography and analyzed by LCMS.[21,22] The percentage of cognate HC/LC pairing was calculated by quantifying the half IgG_1 species. The HC of anti-EGFR, anti-IL-13 and anti-HER2 contain a 'knob' mutation (T366W)[8] whereas the HC of anti-MET, anti-IL-4 and anti-CD3 contain 'hole' mutations (T366S:S368A:Y407V).[8] The asterisk (*) denotes the presence of either 'knob' or 'hole' mutations.

$BsIgG_1$. The amino acid sequence differences between the anti-EGFR and anti-MET antibodies are located entirely within the CDRs plus one additional framework region (FR) residue, V_H 94, immediately adjacent to CDR H3 (Figure S2). The remaining FR, plus C_κ and C_H1 constant domain sequences of these antibodies are identical. CDR L3 and H3 are the CDRs that are most extensively involved at the V_H/V_L domain interface of the anti-MET antibody as evidenced by the X-ray crystallographic structure of the anti-MET Fab complexed with its antigen (Protein Data Bank (PDB) identification code 4K3J).[25] These observations led to the hypothesis that CDR L3 and H3 of the anti-MET antibody may contribute to high bispecific yield for the anti-EGFR/MET $BsIgG_1$. To test this hypothesis, we set out to perform CDR replacement experiments. We chose to replace CDR L3 and

H3 from an anti-CD3 antibody that showed random pairing when combined with anti-HER2. The amino acid sequence of anti-CD3 is different from anti-MET or anti-EGFR within CDR L3 and H3 (Figure S2). The FR region (except V_L 71 and V_H 49, 71, 73, 94) plus C_κ and C_H1 constant domain sequence of anti-CD3 is identical to anti-MET or anti-EGFR antibodies. Consistent with our hypothesis, replacement of both CDR L3 and H3 of the anti-MET antibody with corresponding sequences from an anti-CD3 antibody led to substantial loss of bispecific yield (~85% to 33%, Figure 4(a)). In contrast, replacement of both CDR L3 and H3 of the anti-EGFR arm of the anti-EGFR/MET bispecific resulted in only a small reduction in BsIgG yield (~85% to 75%

Figure 4. Contributions of CDR L3 and H3 to bispecific yield for (a) anti-EGFR/MET and (b) anti-IL-13/IL-4 BsIgG₁. CDR L3 and H3 for one of both arms of each BsIgG₁ were replaced with corresponding sequences from an anti-CD3 antibody to investigate their contribution to BsIgG₁ yield. Data plotted are the mean ± SD of two independent experiments from optimized LC DNA ratios. See Table 1 for additional experimental details.

Figure 4(a)). Replacement of CDR L3 and H3 for both anti-EGFR and anti-MET arms resulted in random HC/LC pairing. These data support the notion that CDR L3 and H3 of anti-MET and anti-EGFR antibodies make major and minor contributions, respectively, to the high bispecific yield observed for the anti-EGFR/MET BsIgG₁. Replacement of CDR L1 and H1 or CDR L2 and H2 from the anti-MET antibody with corresponding anti-CD3 antibody sequences had little to no effect upon bispecific yield for the anti-EGFR/MET BsIgG₁ (Figure S4).

Residues within CDRs L3 and H3 of anti-MET antibody contributing to high bispecific yield of anti-EGFR/MET BsIgG₁

The X-ray crystallographic structure of the anti-MET Fab (PDB accession code 4K3J)[25] revealed contact residues between CDR L3 and H3 (Figure 5(a)) and was used to guide the selection of residues for mutational analysis. Alanine-scanning mutagenesis[26] of anti-MET CDR L3 and H3 was used to map residues contributing to the high bispecific yield of anti-EGFR/MET BsIgG₁ (Figure 5(b)). The V_L Y91A mutation in CDR L3 gave the largest reduction in bispecific yield (84% to 57%) of any of the 12 single alanine mutants tested. As few as two alanine replacements in CDR L3, namely V_L Y91A:Y94A, abolished the high bispecific yield (84% to 23%). Thus, CDR L3 residues V_L Y91 and Y94 appear to make critical contributions to high bispecific yield for the anti-EGFR/MET BsIgG₁. The expression titers of all the mutants were comparable to the parent BsIgG₁ as estimated by the recovered yield from protein A chromatography (data not shown).

CDR L3 and H3 of anti-IL-13 antibody contribute to high bispecific yield for anti-IL-13/IL-4 BsIgG₁

Specific residues in CDR L3 of the anti-MET antibody are important for high bispecific yield for the anti-EGFR/MET BsIgG₁ (see above). We postulated that similar principles may apply to the anti-IL-13 antibody in contributing to high bispecific yield of the anti-IL-13/IL-4 BsIgG₁. An analogous experimental strategy was used to investigate this possibility. One notable difference between these two antibody pairs is that the anti-IL-13 and anti-IL-4 antibodies differ in *both* their CDR and FR sequences (Figure S3) whereas the anti-MET and anti-EGFR antibodies have identical FR sequences (except for V_H 94) and differ in their CDR sequences (Figure S2).

Replacement of CDR L3 and H3 of the anti-IL-13 antibody with corresponding sequences from an anti-CD3 antibody led to substantial loss of bispecific yield of the anti-IL-13/IL-4 BsIgG₁ (~72% to 37%, Figure 4(b) and Figure S3). In contrast, a slight *increase* was observed when CDR L3 and H3 of the anti-IL-4 antibody were replaced in a similar manner (Figure 4(b)). These results suggest that CDR L3 and H3 of the anti-IL-13 antibody contribute to high bispecific yield of the anti-IL-13/IL-4 BsIgG₁.

Alanine-scanning mutational analysis[26] of anti-IL-13 CDR L3 and H3 was used to map residues contributing to the high bispecific yield of anti-IL-13/IL-4 BsIgG₁. The X-ray crystallographic structure of the anti-IL-13 Fab in complex with IL-13 (PDB accession code 4I77)[27] revealed the contact residues
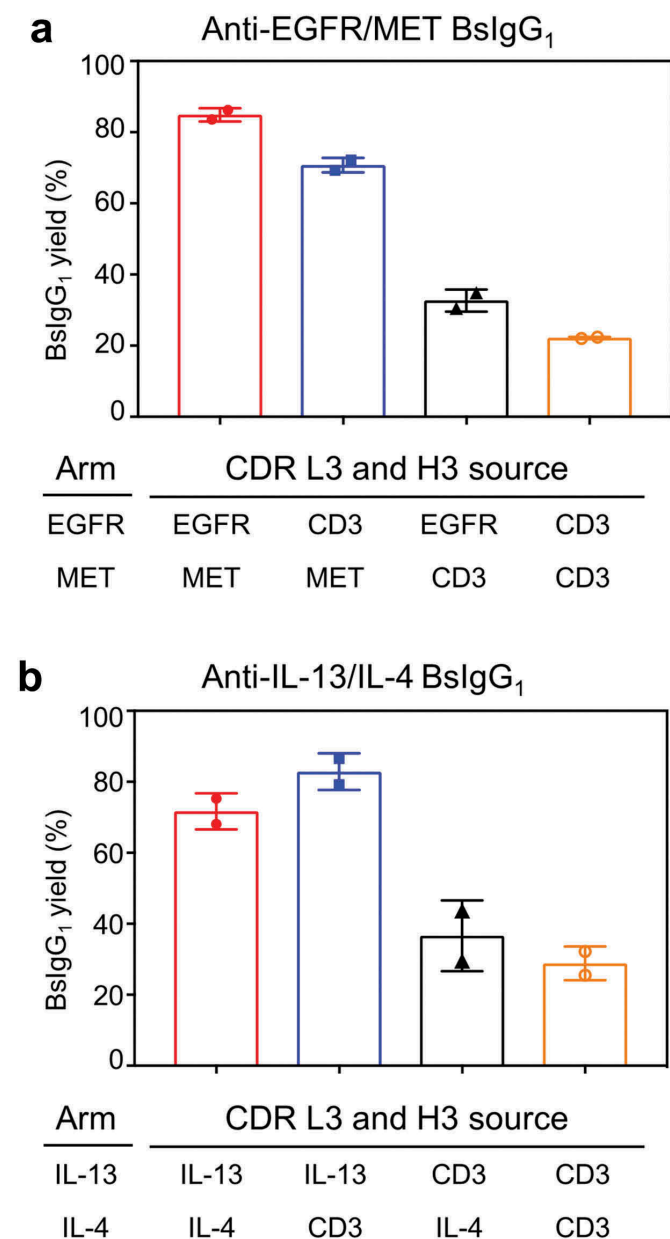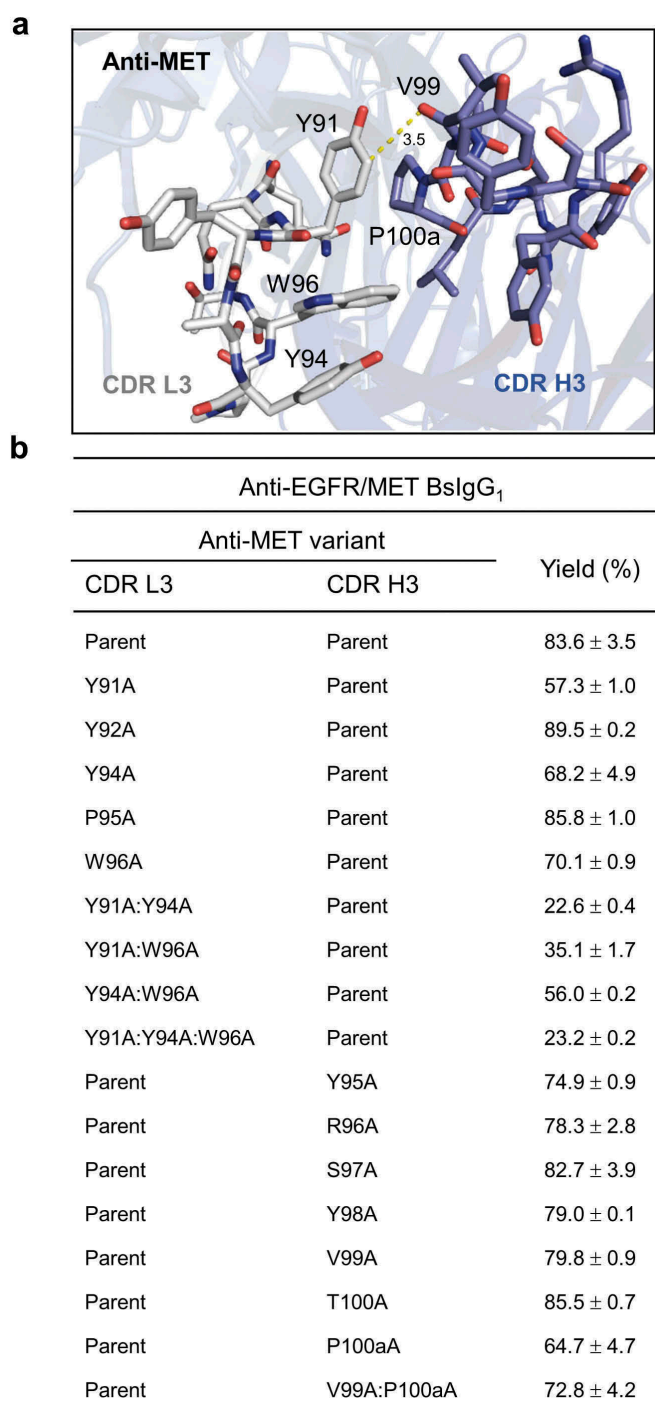
Figure 5. Structure-guided mutational analysis of CDR L3 and H3 contact residues for an anti-MET antibody. (a) X-ray structure of the anti-MET Fab (PDB 4K3J)[25] highlighting CDR L3 and H3 contact residues. (b) Alanine-scanning mutagenesis of CDR L3 and H3 of the anti-MET antibody to identify residues contributing to high bispecific yield of the anti-EGFR/MET BsIgG$_1$. Data shown represent mean ± SD for two independent experiments using optimized LC DNA ratios. See Table 1 for additional experimental details.

Anti-EGFR/MET BsIgG$_1$

Anti-MET variant		Yield (%)
CDR L3	CDR H3	
Parent	Parent	83.6 ± 3.5
Y91A	Parent	57.3 ± 1.0
Y92A	Parent	89.5 ± 0.2
Y94A	Parent	68.2 ± 4.9
P95A	Parent	85.8 ± 1.0
W96A	Parent	70.1 ± 0.9
Y91A:Y94A	Parent	22.6 ± 0.4
Y91A:W96A	Parent	35.1 ± 1.7
Y94A:W96A	Parent	56.0 ± 0.2
Y91A:Y94A:W96A	Parent	23.2 ± 0.2
Parent	Y95A	74.9 ± 0.9
Parent	R96A	78.3 ± 2.8
Parent	S97A	82.7 ± 3.9
Parent	Y98A	79.0 ± 0.1
Parent	V99A	79.8 ± 0.9
Parent	T100A	85.5 ± 0.7
Parent	P100aA	64.7 ± 4.7
Parent	V99A:P100aA	72.8 ± 4.2

between CDR L3 and H3 (Figure 6(a)) and was used to select residues for mutational analysis (Figure 6(b)). The CDR L3 mutation V_L R96A gave the largest reduction in bispecific yield of any of the nine single alanine mutants tested for CDRs L3 and H3 and abolished the high bispecific yield (72% to 29%). As few as two alanine replacements in CDR

H3, namely V_H D95A:P99A, also abolished the high bispecific yield (72% to 26%). The expression titers of all the mutants were comparable to the parent BsIgG$_1$ as estimated by the recovered yield from protein A chromatography (data not shown).

CDR L3 and H3 can increase bispecific yield for BsIgG$_1$

Critical contributions to high bispecific yield can be made by CDR L3 and H3 as judged by both the anti-EGFR/MET and anti-IL-13/IL-4 BsIgG$_1$ studied here. Next, we investigated whether CDR L3 and H3 from these antibodies could be sufficient for providing high bispecific yield for other antibody pairs. We selected two antibody pairs that have low bispecific yield, namely anti-HER2/CD3 (22–24%) (Table 1)[21] and anti-VEGFA/ANG2 (24%).[21] CDR L3 and H3 for one arm each of these two BsIgG$_1$ were replaced with corresponding CDR sequences from either the anti-MET or anti-IL-13 antibodies. A substantial increase in yield of BsIgG$_1$ (from ~24% up to 40–65%) was observed in three of four CDR L3 and H3 recruitment cases for both anti-HER2/CD3 (Figure 7(a)) and anti-VEGFA/ANG2 (Figure 7(b)). Thus, recruitment of CDR L3 and H3 from antibodies with a cognate HC/LC pairing preference can enhance the yield of BsIgG$_1$ with no pairing preference but does not invariably do so.
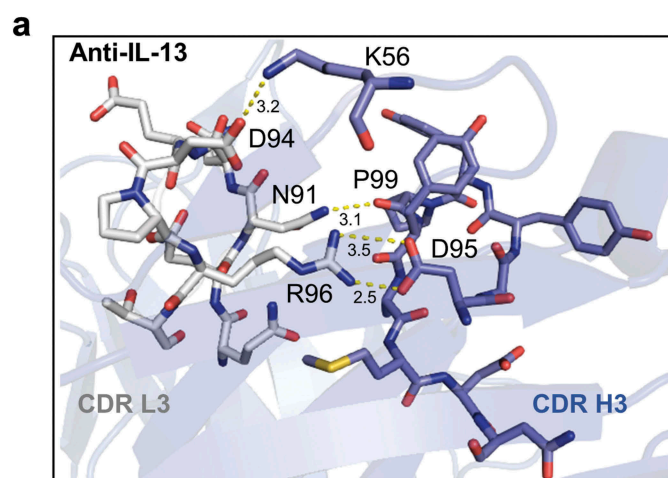
When critical residues for pairing preference for anti-IL-13 were transplanted to the corresponding position in anti-HER2, anti-VEGFA or anti-VEGFC antibodies, some increase in bispecific yield was observed, albeit less than for the parental anti-IL-13/IL-4 BsIgG$_1$ (Table S4). Together, these results suggested that CDR L3 and H3 can impart pairing preference for some, but not all, antibody pairs.

HC-LC inter-chain disulfide bond does not affect the yield of BsIgG$_1$

Previously, we hypothesized that formation of the interchain disulfide bond between the HC and LC acts as a kinetic trap that prevents chain exchange.[21] Here we investigated if the disulfide bond between HC and LC affects the bispecific yield for two BsIgG$_1$ with a pronounced cognate chain preference (anti-EGFR/MET and anti-IL-13/IL-4) and two controls with random HC/LC pairing (anti-HER2/CD3 and anti-VEGFA/VEGFC). BsIgG$_1$ variants lacking the inter-chain disulfide bond were generated using cysteine to serine mutations: LC C214S and HC C220S. Removal of the inter-chain disulfide bond in the engineered variants was verified by SDS PAGE (Figure S5). No clear evidence was found that the inter-chain disulfide bond affects BsIgG$_1$ yield for any of the four antibody pairs tested as judged by native mass spectrometry (Table 2). The yields of BsIgG$_1$ of the parental and the inter-chain disulfide bond lacking variants were similar.

Discussion

BsAb, including BsIgG, holds significant promise as next-generation antibody drugs prompting much interest in the development of robust and efficient production methods for

Figure 6. Structure-guided mutational analysis of CDR L3 and H3 contact residues for an anti-IL-13 antibody. (a) X-ray structure of the anti-IL-13 Fab (PDB 4I77)[27] highlighting CDR L3 and H3 contact residues. (b) Alanine-scanning mutagenesis of CDR L3 and H3 of anti-IL-13 to identify residues contributing to high bispecific yield of anti-IL-13/IL-4 BsIgG$_1$. Data shown represent mean ± SD for two independent experiments using optimized LC DNA ratios. See Table 1 for additional experimental details.

b

Anti-IL-13/IL-4 BsIgG$_1$

Anti-IL-13 variant		Yield (%)
CDR L3	CDR H3	
Parent	Parent	71.8 ± 1.6
N91A	Parent	65.4 ± 2.1
N92A	Parent	69.7 ± 1.1
D94A	Parent	78.1 ± 3.3
R96A	Parent	28.7 ± 1.4
N91A:D94A	Parent	68.7 ± 3.5
D94A:R96A	Parent	24.8 ± 2.1
N91A:D94A:R96A	Parent	36.8 ± 0.1
Parent	D95A	55.9 ± 0.1
Parent	Y97A	77.0 ± 1.9
Parent	Y98A	63.7 ± 0.7
Parent	P99A	72.5 ± 1.3
Parent	Y100A	55.7 ± 2.8
Parent	D95A:P99A	26.1 ± 2.9

Figure 7. Recruitment of CDR L3 and H3 from either anti-MET or anti-IL-13 antibodies into other antibody pairs to determine the effect upon BsIgG$_1$ yield. CDR L3 and H3 of (a) anti-HER2 and anti-CD3 antibodies and (b) anti-VEGFA and anti-ANG2 antibodies were replaced with CDR L3 and H3 of either the anti-MET or anti-IL-13 antibodies. The data presented are from optimized LC DNA ratios. The BsIgG$_1$ yield was determined as for Table 1.

these complex molecules. BsIgG have been efficiently produced in single host cells by multiple strategies, including a combination of Fab and Fc engineering to favor cognate over non-cognate HC/LC pairing and HC heterodimerization over homodimerization, respectively.[16–21,24] Assessing the success of such Fab engineering requires comparison of variants with and without Fab mutations. For example, we transiently co-expressed two different HC and LC in mammalian cells, 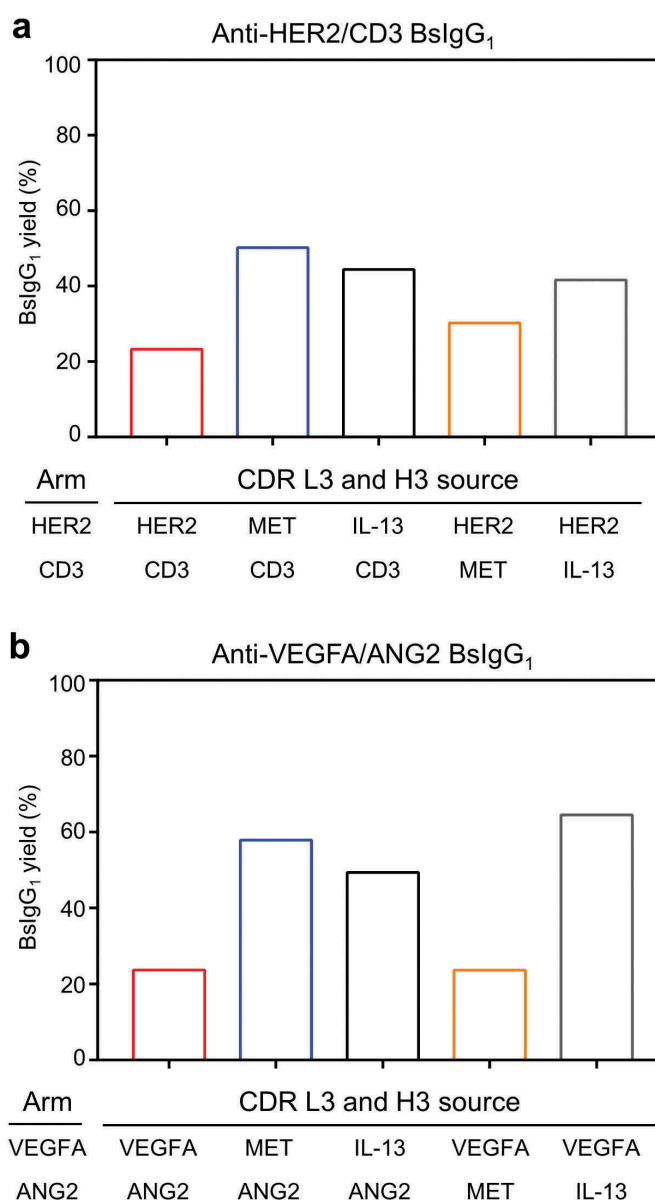purified the mixture of different IgG species, and then determined the percentage of BsIgG present (defined here as BsIgG yield) by high-resolution LCMS.[21,22] Two antibody pairs gave >65% BsIgG$_1$ with KIH mutations for HC hetero-dimerization, but *without* Fab mutations to favor cognate HC/LC pairing.[21] This observation suggests the existence of a strong intrinsic preference for cognate HC/LC pairing for some antibody pairs that has also been reported by other groups.[16,24] We sought to better understand cognate HC/LC pairing preference, including strength and frequency of occurrence of this phenomenon. Additionally, this chain pairing preference information was used to reduce the number of Fab arm mutations needed for the efficient production of BsIgG$_1$ in single host cells for some antibody pairs.

Table 2. Mutational analysis suggests that the disulfide bond between HC and LC does not affect BsIgG$_1$ yield. Experimental conditions were similar to those described in Table 1 legend except that the quantification of BsIgG$_1$ utilized native LCMS (see Materials and Methods).[28] Variants lacking the interchain disulfide bond were constructed using C$_L$ C214S and C$_H$1 C220S mutations. Data shown are the mean ± SD for three biological replicates using optimized DNA LC ratios.

	BsIgG$_1$ yield (%)	
BsIgG$_1$	Parent with HC/LC disulfide bond	Variant without HC/LC disulfide bond
Anti-EGFR/MET	81.1 ± 1.4	82.8 ± 2.6
Anti-IL-13/IL-4	73.3 ± 4.5	75.1 ± 0.8
Anti-HER2/CD3	24.5 ± 0.8	27.0 ± 2.4
Anti-VEGFA/VEGFC	28.8 ± 5.9	38.0 ± 6.0

In this study, we extended prior work on understanding cognate HC/LC preferences by expanding upon the small number of previously reported BsIgG examples.[16,21,24] We produced 99 different BsIgG$_1$ with KIH HC, but *without* Fab mutations. Strikingly, non-random HC/LC pairing (>30% yield BsIgG$_1$) was observed for most (>80%) antibody pairs, with high (>65%) BsIgG$_1$ yield seen for one-third of antibody pairs (Table 1). The observed differences in BsIgG$_1$ yield for different antibody pairs are presumably driven by V$_H$ and V$_L$ sequence differences because most antibodies evaluated here share identical human C$_H$1 and κ C$_L$ sequences. *A priori*, either one or both Fab arms of a bispecific may exhibit a cognate HC/LC pairing preference contributing to the high yield of BsIgG$_1$. Evidence of each of these possibilities was found from co-expression of different HC in the presence of competing cognate and non-cognate LC (Figure 3).

One caveat to the interpretation of CDR replacement experiments discussed here is that the observed effect upon BsIgG$_1$ yield is a combination of both removal of the original CDRs as well as the newly installed CDR. This inherent methodological limitation notwithstanding, the diverse data types present in this study (three chain co-expression, CDR replacements and CDR point mutations) are consistent with the notion that CDR L3 and H3, and specific residues within these CDRs can contribute to cognate HC/LC pairing preference. This hypothesis appears plausible since CDR L3 and H3 interactions can contribute to V$_H$/V$_L$ domain interfaces as evidenced by X-ray crystallographic studies.[16,17,20] Replacements of CDR L3 and H3 and mutations therein are likely to affect antigen binding. Since the major focus of this study was to understand the molecular basis of HC/LC chain pairing preferences, we did not measure the affinities for antigen binding. Optimization and utilization of the molecular understanding of HC/LC chain pairing preference for antigen binding are beyond the scope of this investigation. Removal of the inter-chain disulfide bond by mutagenesis had no clear effect upon BsIgG$_1$ yield (Table 2), suggesting HC/LC pairs are not readily prone to exchange.

BsIgG designed for efficient production in single cells has in several cases utilized mutations at all four Fab domain interfaces, i.e., V$_H$/V$_L$ and C$_H$1/C$_L$ in both Fab arms.[16,17,20,21] Extensively engineered proteins can be successfully developed as drugs, as exemplified by the BsIgG$_1$, emicizumab.[4,5,29–31] This achievement notwithstanding, reducing the number of Fab arm mutations for assisting BsIgG production in single

cells is possible for many antibody pairs and may be desirable as discussed below. The many potential benefits of introducing mutations to enhance antibody and other protein drug candidates[2,32] need to be weighed against possible untoward effects that can occasionally occur. Undesirable attributes occasionally encountered with engineered proteins include off-target binding and faster pharmacokinetic clearance,[33] reduced thermostability,[8,20,34] reduced affinity for binding partners,[34,35] introduction of chemical degradation 'hotspots',[36] and higher incidence of anti-drug antibodies (ADA) elicited in patients.[37] Many such risks can be identified and mitigated through preclinical experimentation as elegantly demonstrated by the elimination of untoward off-target binding of an affinity-matured anti-RSV antibody.[33] Other developability risks with engineered proteins are more challenging to assess preclinically, such as the likelihood for eliciting neutralizing ADAs in patients.[38,39]

Ideally, BsIgG would be produced in single cells *without* the need for Fab mutations as recently demonstrated to be possible for one antibody pair.[24] Here we identified two additional examples of near quantitative (>90%) formation of BsIgG$_1$ using KIH HC but *without* Fab mutations (Table 1). However, such a high stringency, inherent HC/LC pairing preference was rare and observed here in only ~2% of the antibody pairs we surveyed. The development of methods to identify or screen for such antibody pairs may be helpful in constructing BsIgG and other BsAb. The use of engineered disulfide bonds to enhance the assembly of BsIgG$_1$ was first shown in the context of promoting HC heterodimerization.[9] More recently, the native disulfide bond between C$_H$1 and C$_L$ domains was replaced with an engineered disulfide between these domains for enhancing the production of BsIgG$_1$ in single cells.[18] In this case, efficient assembly of BsIgG$_1$ was achieved with as few as four amino-acid replacements.[18] The native disulfide bond between HC and LC has also been replaced with an engineered disulfide between V$_H$ and V$_L$ for robust one cell production of BsIgG.[19] Comparison of different sites suggests that installing the engineered disulfide at the V$_H$/V$_L$ rather than C$_H$1/C$_L$ interface is the preferred site to prevent non-cognate V$_H$/V$_L$ pairing.

Initial studies to assist BsIgG production in single cells by engineering the C$_H$1/C$_L$ interfaces alone met with limited success and led to the idea that variable domains can dominate HC/LC pairing preference.[16] We later demonstrated that a different set of C$_H$1/C$_L$ mutations increased the BsIgG$_1$ yield somewhat for an anti-HER2/CD3 BsIgG$_1$ with random HC/LC pairing.[21] More recently, Bonisch et al. demonstrated that alternative C$_H$1/C$_L$ mutations alone could promote near quantitative formation of BsIgG$_1$ for two antibody pairs with a strong cognate chain pairing preference.[24] Here, we further developed this concept by showing that antibodies with a strong cognate pairing preference occur commonly, comprising one-third of the antibody pairs surveyed here (Table 1). Additionally, we demonstrate that for most antibody pairs (9 of 11) with a strong (>65%) HC/LC pairing preference, C$_H$1/C$_L$ mutations (same as previously used)[21] in conjunction with KIH HC are often sufficient to support near quantitative yield of BsIgG$_1$ (Figure 2). Thus, the number of Fab arm mutations to facilitate efficient BsIgG$_1$ assembly was reduced from a

minimum of eight or more in our previous study[21] to just four in some cases (Figure 2.) or even zero in rare (~2%) instances (Table 1).

In summary, this study demonstrates that a cognate HC/LC pairing preference in producing BsIgG in single cells is a common phenomenon that is highly dependent upon the specific antibody pair. Mechanistically, this chain pairing preference can be strongly influenced by residues in CDR L3 and H3. Practically, this cognate HC/LC pairing preference was used to reduce the number of Fab mutations needed to drive the production of BsIgG$_1$, and potentially BsIgG of other isotypes, in single cells.

Materials and methods

Antibody construct design and synthesis

All antibodies in this work are numbered using the Kabat[40] and EU[41] numbering systems for variable and constant domains, respectively. Antibody constructs were generated by gene synthesis (GeneWiz), and wherever applicable, sub-cloned into the expression plasmid (pRK5) as described previously.[21] All antibody HC in this study were aglycosylated (N297G mutation) and with the carboxy-terminal lysine deleted (ΔK447) to reduce product heterogeneity, and thereby facilitate accurate quantification of BsIgG$_1$ by LCMS.[21,22] The two-component HC of all BsIgG$_1$ in this study were engineered to contain either a 'knob' mutation (T366W)[8] in the first listed antibody or 'hole' mutations (T366S:L368A:Y407V)[8] in the second listed antibody to facilitate HC heterodimerization.[8]

For a few of the BsIgG$_1$ in this study, FR mutations were judiciously made to provide sufficient mass difference between correctly paired and mispaired BsIgG$_1$ species for more accurate quantitation by LCMS analysis. The mass difference needed for accurate quantification of bispecific IgG yield is ≤118 Da.[22] Specifically, the antibodies and mutations were anti-HER2 V$_L$ R66G when combined with anti-CD3 or variants (Figure 7(a)), anti-IL-1β or anti-GFRα (Table 1); anti-VEGFA V$_L$ F83A when combined with anti-ANG2 or variants (in Figure 7(b)); anti-CD3 V$_L$ N34A:F83A when combined with anti-Factor D 25D7 v1 or anti-IL-33; anti-RSPO3 V$_L$ F83A, when combined with anti-CD3; anti-EGFR V$_L$ F83A when combined with anti-SIRPα or anti-Factor D 20D12 v1; plus anti-IL-4 V$_L$ N31A:F83A when combined with anti-GFRα1 (Table 1 or Figure 1). The chosen residues had no detectable impact on BsIgG$_1$ yield based upon comparison with parental antibodies.

Antibody expression and purification

All BsIgG$_1$ were transiently expressed in Expi293F cells as described previously.[21] Four plasmids corresponding to the two LC and two HC were co-transfected into Expi293F cells. The LC DNA was varied for each experiment and the highest bispecific yield with the optimal LC:HC ratio was reported as described previously.[21] Briefly, the LC:HC ratios used in this study were 1:3, 1:2, 1:1, 2:1 or 3:1. The ratio of the two HC was not optimized and fixed at 1:1. The optimized LC:HC DNA ratios from the four chain co-expression experiments were used for

experiments in Figure 3. The LC1:LC2 DNA ratios used were 1:1 for all except 1:2 for EGFR:MET. The transfected cell culture (30 mL) was grown for 7 days at 37°C with shaking. BsIgG$_1$ from the filtered cell culture supernatants were purified in a high throughput fashion by Toyopearl AF-rProtein A affinity chromatography (Tosoh Bioscience). Impurities such as aggregates and half IgG$_1$ were removed by SEC using a Zenix-C SEC-300 column (10 mm × 300 mm, 3 μm particle size, Sepax Technology). The IgG$_1$ concentration was calculated using an extinction coefficient A$^{0.1\%}_{280nm}$ of 1.4. Purification yield was estimated after protein A chromatography by multiplying the protein concentration with elution volume.

Analytical characterization of BsIgG$_1$ by SEC HPLC

BsIgG$_1$ samples (20 μL) were chromatographed under isocratic conditions on a TSKgel SuperSW3000 column (4.6 × 150 mm, 4 μm) (Tosoh Bioscience) connected to a Dionex UltiMate 3000 HPLC (Thermo Scientific). The mobile phase was 200 mM potassium phosphate and 250 mM potassium chloride at pH 7.2 with a flow rate of 0.3 mL/min with absorbance measurement at a wavelength of 280 nm.

BsIgG$_1$ yield determination by high-resolution LCMS

Quantification of BsIgG$_1$ yield (intensity of correctly paired LC species over all three mispaired IgG$_1$ species) was performed using a Thermo Exactive Plus Extended Mass Range Orbitrap mass spectrometer as described previously and assumes no response bias amongst the different mass peaks.[22]

For denaturing mass spectrometry, samples (3 μg) were injected onto a MAbPac reversed-phase liquid chromatography column (Thermo Fisher Scientific, 2.1 mm × 50 mm) heated to 80°C using a Dionex Ultimate 3000 RSLC system. A binary gradient pump was used to deliver solvent A (99.88% water containing 0.1% formic acid and 0.02% trifluoroacetic acid) and solvent B (90% acetonitrile containing 9.88% water plus 0.1% formic acid and 0.02% trifluoroacetic acid) as a gradient of 20% to 65% solvent B over 4.5 min at 300 μL/min. The solvent was step-changed to 90% solvent B over 0.1 min and held at 90% for 6.4 min to clean the column. Finally, the solvent was step-changed to 20% solvent B over 0.1 min and held for 3.9 min for re-equilibration. Samples were analyzed online via electrospray ionization into the mass spectrometer using the following parameters for data acquisition: 3.90 kV spray voltage; 325°C capillary temperature; 200 S-lens RF level; 15 sheath gas flow rate and 4 AUX gas flow rate in ESI source; 1,500 to 6,000 m/z scan range; desolvation, in-source CID 100 eV, CE 0; resolution of 17,500 at m/z 200; positive polarity; 10 microscans; 3E6 AGC target; fixed AGC mode; 0 averaging; 25 V source DC offset; 8 V injection flatapole DC; 7 V inter-flatapole lens; 6 V bent flatapole DC; 0 V transfer multipole DC tune offset; 0 V C-trap entrance lens tune offset; and trapping gas pressure setting of 2.

For native mass spectrometry, samples (10 μg) were injected onto an Acquity UPLC BEH SEC column (Waters, 4.6 mm × 150 mm) heated to 30°C using a Dionex Ultimate 3000 RSLC system. Isocratic chromatography runs (10 min)

utilized an aqueous mobile phase containing 50 mM ammonium acetate at pH 7.0 with a flow rate of 300 μL/min.

Samples were analyzed online via electrospray ionization into the mass spectrometer using the following parameters for data acquisition: 4.0 kV spray voltage; 320°C capillary temperature; 200 S-lens RF level; 40 sheath gas flow rate and 20 AUX gas flow rate in ESI source; 300 to 20,000 m/z scan range; desolvation, in-source CID 100 eV, CE 0; resolution of 17,500 at m/z 200; positive polarity; 10 microscans; 1E6 AGC target; fixed AGC mode; 0 averaging; 25 V source DC offset; 8 V injection flatapole DC; 7 V inter-flatapole lens; 6 V bent flatapole DC; 0 V transfer multipole DC tune offset; 0 V C-trap entrance lens tune offset; and trapping gas pressure setting of 2.

Acquired mass spectral data were analyzed using Protein Metrics Intact Mass and Thermo BioPharma Finder 3.0 software. The signal intensity of the correctly paired LC species from the deconvolved spectrum of each sample was used for quantification relative to the three mispaired IgG_1 species. HC homodimers and half IgG were either undetectable or present in trace amounts and excluded from the calculations. The correctly LC paired $BsIgG_1$ were estimated from the isobaric mixture of $BsIgG_1$ and the double LC mispaired IgG_1 by using the algebraic formula described previously.[22]

Abbreviations

BsAb	bispecific antibodies
BsIgG	bispecific IgG
CDR	complementarity-determining region
EMR	extended mass range
FR	framework region
HC	heavy chain
KIH	knob-into-hole
LC	light chain
LCMS	liquid chromatography-mass spectrometry
NA	not applicable
ND	not detected
PDB	Protein Data Bank
SD	standard deviation
SEC	size-exclusion chromatography

Disclosure of potential conflicts of interest

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