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Insights into the molecular basis of a bispecific antibody's target selectivity

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Abbreviations: KD, dissociation constant; PBS, phosphate buffered saline; bsAb, bispecific antibody; ADCC, antibody-dependent cell-mediated cytotoxicity

Bispecific antibodies constitute a valuable class of therapeutics owing to their ability to bind 2 distinct targets. Dual targeting is thought to enhance biological efficacy, limit escape mechanisms, and increase target selectivity *via* a strong avidity effect mediated by concurrent binding to both antigens on the surface of the same cell. However, factors that regulate the extent of target selectivity are not well understood. We show that dual targeting alone is not sufficient to promote efficient target selectivity, and report the substantial roles played by the affinity of the individual arms, overall avidity and valence. More particularly, various monovalent bispecific IgGs composed of an anti-CD70 moiety paired with variants of the anti-CD4 mAb ibalizumab were tested for preferential binding and selective depletion of $CD4^+$/$CD70^+$ T cells over cells expressing only one of the target antigens that resulted from antibody dependent cell-mediated cytotoxicity. Variants exhibiting reduced CD4 affinity showed a greater degree of target selectivity, while the overall efficacy of the bispecific molecule was not affected.

Introduction

Antibodies have had a dramatic impact on biomedical research, and antibody therapeutics for numerous indications, including cancer, autoimmunity, inflammation and infectious disease, are enjoying tremendous success in the clinic.[1-4] However, their monospecific configuration also restricts overall therapeutic potential owing to the simultaneous deregulation of several mediators in many diseases.[5-7] Bispecific antibodies (bsAb) enable simultaneous engagement of 2 targets and offer the potential of greater therapeutic efficacy while overcoming major escape mechanisms seen in mono-targeted therapy.[8-13] It is commonly believed that the dual targeting of 2 antigens on the same cell leads to improved target selectivity over normal tissues that express only one or low levels of both target antigens. This effect is thought to be dependent on the avidity component mediated by the concurrent binding of the bsAb to both antigens on the same cell.[14-17] However, such claims are often made irrespective of 2 critical factors: 1) the intrinsic binding affinity of the 2 separate binding arms, and 2) the configuration of the bsAb binding domains, namely monovalent *vs*. bivalent. While it has been speculated that altering the affinity of the separate arms may increase the window of selectivity without impairing targeting capabilities,[16] the relative importance of affinity, avidity and valence in relation to the ability of a bsAb to confer target selectivity is not well understood. We set out to shed light on these factors.

We recently reported the development of a novel monovalent bispecific IgG platform ('DuetMab'), and showed the ability of such a molecule composed of an anti-CD4 (ibalizumab[18]) and anti-CD70 (2H5) arm[19] to concurrently bind CD4 and CD70 on the surface of the same cell.[20] This revealed improved binding selectivity of the anti-CD4/CD70 DuetMab to a target population of $CD4^+$/$CD70^+$ T cells in a cell mixture containing 'non-target' lymphocytes expressing only 1 of the 2 antigens. However, although binding to $CD4^-$/$CD70^+$ B cells was virtually eliminated, substantial binding to $CD4^+$/$CD70^-$ T cells remained.

In an effort to understand how target selectivity may be further improved, we generated an array of affinity-reduced variants of the anti-CD4 ibalizumab mAb. We then assessed the target selectivity of the corresponding anti-CD4/CD70 DuetMab variants by measuring their ability to preferentially bind to, and deplete via antibody-dependent cell-mediated cytotoxicity (ADCC), the target

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$CD4^+/CD70^+$ T cells over non-target cells. We show for the first time that the intrinsic affinity of the separate arms plays a pivotal role in the ability of a bsAb to achieve target selectivity. We further demonstrate how avidity and binding valence regulate selective cell targeting. These findings have important implications for the development of clinically relevant bsAbs.

Results

Functional characterization of anti-CD4/CD70 DuetMab

To determine the selectivity of the parental anti-CD4/CD70 DuetMab (**Fig. 1**), cell populations expressing either both ($CD4^+/CD70^+$ T cells) or only one ($CD4^+/CD70^-$ T cells and $CD4^-/CD70^+$ B cells, so-called 'non-target') antigen were pre-stained with different tracer dyes, combined at equal ratios and incubated with serial dilutions of anti-CD4/CD70 DuetMab prior to analysis by flow-cytometry. This assay design enables a predictive examination of selective targeting as it simulates a physiological condition where target and non-target cells co-exist. Consistent with our previously published data,[20] anti-CD4/CD70 DuetMab exhibited superior binding to the target $CD4^+/CD70^+$ T cells over the 2 non-target cells (**Fig. 2A**). However, while the selectivity for $CD4^+/CD70^+$ T cells over the $CD4^-/CD70^+$ B cells was very significant, the preference over the non-target $CD4^+/CD70^-$ T cells was more modest.

In an effort to understand whether such binding properties translated to functional selectivity, we tested the ability of the anti-CD4/CD70 DuetMab to mediate selective ADCC depletion of $CD4^+/CD70^+$ T cells using the same cell mix as above. We employed ADCC because it provides the most direct correlation between killing and the amount of Fc domains associated with the cell. More particularly, depletion was the readout because it is the only method that allows simultaneous ADCC analysis of multiple cells while distinguishing the different populations. KC1333 natural killer (NK) cells were used as effector cells and cell cytotoxicity was determined by means of flow-cytometry. To facilitate comparative analysis of the data, the ADCC activity mediated by anti-CD4/CD70 DuetMab against $CD4^+/CD70^+$ T cells was normalized to 100% (**Fig. 2B**). In agreement with binding data, at a concentration of 2.5 nM, the cytotoxic activity mediated against $CD4^+/CD70^+$ T cells was estimated at >95% while the non-target cytotoxicity against $CD4^+/CD70^-$ T cells and $CD4^-/CD70^+$ B cells was estimated at ~45 and 5%, respectively (**Fig 2B**). Receptor density analysis showed that CD4 levels on the 2 T cell populations used in these experiments was comparable (**Table 1**); therefore, differences in CD4 expression cannot be invoked for the significant targeting of $CD4^+/CD70^-$ cells. We next determined the binding kinetics of anti-CD4/CD70 DuetMab to CD4 and CD70 (**Table 2**). The monovalent affinity of the anti-CD4 and -CD70 arm to CD4 and CD70 was estimated at 0.9 and 25 nM, respectively.

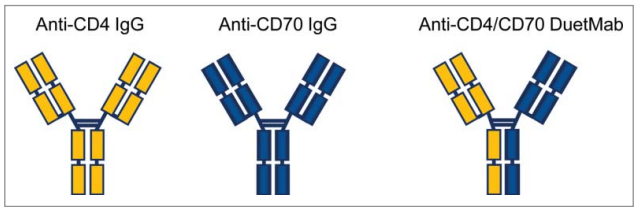

Figure 1. Schematic diagram showing conventional bivalent monospecific anti-CD4 and anti-CD70 IgGs along with anti-CD4/CD70 monovalent bispecific DuetMab.

Taken together, our findings suggest that significant non-target ADCC can be induced by a monovalent bispecific molecule, provided the affinity of a single arm is high enough (~1 nM in our case). From this follows that dual targeting alone is not always sufficient to achieve efficient target selectivity.

Generation and characterization of affinity-reduced anti-CD4 variants

To determine the relationship between affinity of the anti-CD4 arm and target selectivity of the anti-CD4/CD70 DuetMab, we constructed an array of affinity-reduced variants. Based on information captured from the structure of ibalizumab bound to the first 2 domains of CD4 (D1-D2),[21] we carried out alanine mutagenesis to core contact residues in complementarity-determining region (CDR)H3 and L3 that primarily interact with CD4 BC and FG loops. We constructed 13 IgG variants carrying either a single mutation in CDRH3 or L3 or a combination of mutations in both CDRs. Their binding kinetics to CD4 were determined by Octet analysis (**Table 2**). Though affinity reductions ranging from ~2-100-fold were observed, no significant change was seen in association rates (K_{on}). Those variants that exhibited either minor binding differences from the parental IgG or ablated binding were omitted from further characterization. More particularly, we selected and converted into a DuetMab format 5 variants exhibiting an ~20-100-fold reduction in affinity compared with the parental sequence. These variants were expressed and purified from mammalian cells as previously described.[20] Their purity and oligomeric state were assessed using a BioAnalyzer (**Fig. S1A**) and by size exclusion chromatography (SEC) (**Fig. S1B**). The expected mass and homogeneity of the intact molecules were confirmed by reversed-phase high performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS) as shown in **Figure S1C**. The corresponding DuetMab variants retained the relative intrinsic affinity and ranking of the IgGs from which they were derived (**Table 2**).

CD4 affinity-reduced DuetMabs exhibit improved binding selectivity

To assess whether affinity modulation of the anti-CD4 arm leads to improved binding selectivity, we tested the ability of the DuetMab variants to preferentially bind to the target $CD4^+/CD70^+$ T cells from a cell mixture also containing $CD4^+/CD70^-$ T cells and $CD4^-/CD70^+$ B cells. Binding of all variants to $CD4^+/CD70^+$ T cells was not substantially impaired upon reduction of CD4 affinity (**Fig. 3A**). We attribute this result to the avidity mediated by concurrent binding of the DuetMab to CD4 and CD70 on the same cell. This is in agreement with the previous findings that significant avidity could indeed emanate

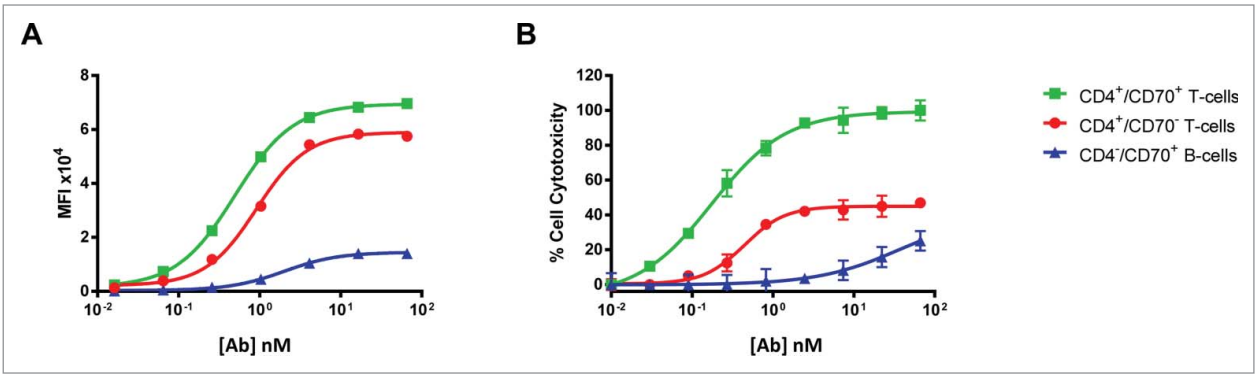

Figure 2. Cell binding and ADCC activity of anti-CD4/CD70 DuetMab. (**A**) Anti-CD4/CD70 DuetMab exhibits preferential cell binding to $CD4^+/CD70^+$ T cells *via* concurrent engagement to CD4 and CD70 on a single cell. (**B**) Anti-CD4/CD70 DuetMab preferentially kills $CD4^+/CD70^+$ T cells as measured by ADCC. Each point represents the mean value of triplicate wells and the standard deviation is represented by error bars.

from multi-specific or -valent molecules.[22,23] However, all variants exhibited improved binding selectivity over the parental DuetMab as indicated by their significant reduction in binding to the non-target $CD4^+/CD70^-$ cells (**Fig. 3B**). Finally, since the affinity of the CD70 arm was not modified, all variants had similarly low binding properties to B cells compared with the parental DuetMab (**Fig. 3C**). Taken together, our data show that the binding selectivity of a monovalent bispecific molecule is directly influenced by the intrinsic affinity of its individual arms.

CD4 affinity-reduced DuetMabs exhibit improved ADCC selectivity

To determine whether improved binding selectivity can be harnessed to enhance functional selectivity, DuetMab variants were tested for their ability to elicit selective ADCC depletion of the target $CD4^+/CD70^+$ T cells over the non-target population of $CD4^+/CD70^-$ T cells and $CD4^-/CD70^+$ B cells. Consistent with cell binding results, the CD4 affinity-reduced DuetMab variants mediated a greater degree of selectivity compared with the parental DuetMab as reflected by a significant reduction in the non-target cytotoxicity on $CD4^+/CD70^-$ T cells (**Fig. 4A**). In particular, the level of selectivity was inversely proportional to the reduced intrinsic affinity to CD4. To allow comparative analysis of the data, we calculated the concentrations of antibody needed to induce 20% cytotoxicity on target ($CD4^+/CD70^+$) and non-target ($CD4^+/CD70^-$) T cells. As shown in **Table 3**, 0.5 nM of the parental DuetMab was required to achieve the targeted non-target lysis level. In comparison, 2 to 60 nM of various variants were required to achieve the same level of cytotoxicity. Importantly, when tested against the target $CD4^+/CD70^+$ T cells, all variants were able to achieve 100% selective ADCC (**Fig. 4A**), and kept similar potency to the parental DuetMab (**Table 3**). To confirm that the improved selectivity observed with the affinity-modulated DuetMab variants was not affected by the E:T ratio, we compared the reporter-based cytotoxic activity triggered by the parental and anti-CD4 $V\kappa Y94A+V_HY99A$/CD70 DuetMab at saturating concentration (1 μg/ml) and at E:T ratios of 1:1, 5:1, 25:1, and 50:1. As shown in **Figure 4B**, selectivity was maintained at all E:T ratios. We conclude that decreasing the affinity of a monovalent bispecific molecule's arm could significantly limit its activity against non-target cells without impairing its potency toward the targeted population.

Table 1. CD4 and CD70 receptor density on human lymphocytes

Cell	CD4	CD70
$CD4^+/CD70^+$ T cells	4.6×10^4	5.2×10^4
$CD4^+/CD70^-$ T cells	3.8×10^4	$< 10^2$
$CD4^-/CD70^+$ B cells	$< 10^2$	3.1×10^4

Effect of avidity and valence on target selectivity

To elucidate the role of concurrent bivalent engagement in the ability of a DuetMab to exhibit target selectivity, we generated 2 monospecific DuetMab molecules; the first was composed of the anti-CD4 $V\kappa Y94A+V_HY99A$ variant paired with a isotype control ('NMGC') whereas the second was composed of the anti-CD70 arm paired with NMGC. As shown in **Figure 5A**, when tested on target $CD4^+/CD70^+$ T cells alone, a 1:1 mixture of the 2 monospecific DuetMabs demonstrated reduced binding compared with the bispecific anti-CD4$V\kappa Y94A+V_HY99A$/CD70 DuetMab variant. Similarly, the same mixture was significantly less potent than the bispecific anti-CD4 $V\kappa Y94A+V_HY99A$/CD70 DuetMab variant in its ability to induce ADCC (**Fig. 5B**). Finally, we compared the levels of cytotoxicity mediated by CD4 affinity-reduced variants against non-target $CD4^+/CD70^-$ T cells alone when formatted either as a monovalent anti-CD4/CD70 DuetMab or bivalent anti-CD4 IgG. As shown in **Figure 5C**, the CD4 affinity-reduced variants induced a more potent non-target ADCC when formatted as bivalent IgGs compared to their monovalent DuetMab counterparts. This resulted in a loss of their previously described improved selectivity. These results suggest that an increase in valence (i.e., avidity) may correlate with an overall increase in activity against non-target cells. Because we have only shown this effect using the CD4 system, the universality of this observation remains to be seen.

Table 2. Binding affinity of IgG and DuetMab to CD4 and CD70

	IgG			DuetMab		
Antibody	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_D (nM)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_D (nM)
Parental (against CD70)[a]	2.2×10^5	5.1×10^{-3}	23	2.0×10^5	4.9×10^{-3}	25
Parental (against CD4)[b]	2.1×10^5	1.8×10^{-4}	0.8	2.8×10^5	2.6×10^{-4}	0.9
VκY94A[b]	2.2×10^5	3.1×10^{-4}	1.4	ND	ND	
VκS93A[b]	2.2×10^5	3.6×10^{-4}	1.6	ND	ND	
V_HD97A[b]	1.6×10^5	6.4×10^{-4}	4.1	ND	ND	
VκY92A[b]	1.2×10^5	1.8×10^{-3}	15	2.0×10^5	1.9×10^{-3}	10
VκY91A[b]	1.7×10^5	4.2×10^{-3}	25	2.8×10^5	4.7×10^{-3}	17
VκR95A[b]	3.0×10^5	1.6×10^{-2}	55	5.4×10^5	2.3×10^{-2}	42
VκR95A+V_HD97A[b]	2.7×10^5	1.8×10^{-2}	65	5.6×10^5	3.5×10^{-2}	63
V_HY99A[b]	2.8×10^5	2.0×10^{-2}	72	ND	ND	
VκS93A+V_HY99A[b]	2.7×10^5	2.0×10^{-2}	74	ND	ND	
VκY94A+V_HY99A[b]	2.7×10^5	2.1×10^{-2}	77	5.2×10^5	3.6×10^{-2}	70
VκY92A+V_HY99A[b]	ND	ND	ND[c]	ND	ND	
VκY91A+V_HY99A[b]	ND	ND	ND[c]	ND	ND	
VκR95A+V_HY99A[b]	ND	ND	ND[c]	ND	ND	

Kinetic measurements to soluble monomeric forms of CD4 and CD70 were carried out using an Octet384 instrument. The dissociation constants, K_D, were calculated as the ratio of k_{off}/k_{on} from a non-linear fit of the data

[a] Binding measured against CD70

[b] Binding measured against CD4

[c] No measurable binding could be seen

ND: not determined

Discussion

Bispecific antibodies represent an emerging class of biologics. Their dual targeting properties offer the potential for greater therapeutic efficacy and hold the promise of improved target selectivity. In this study, we dissected the respective roles of affinity, avidity and valence in the capacity of a monovalent bispecific molecule to induce target selectivity. In particular, we have shown that: 1) dual targeting alone is not sufficient for efficient target selectivity; 2) target selectivity is clearly influenced by the intrinsic affinity of the separate binding arms and can be improved by CDR engineering; 3) improved binding selectivity translates to enhanced functional selectivity; 4) selectivity engineering can be carried out

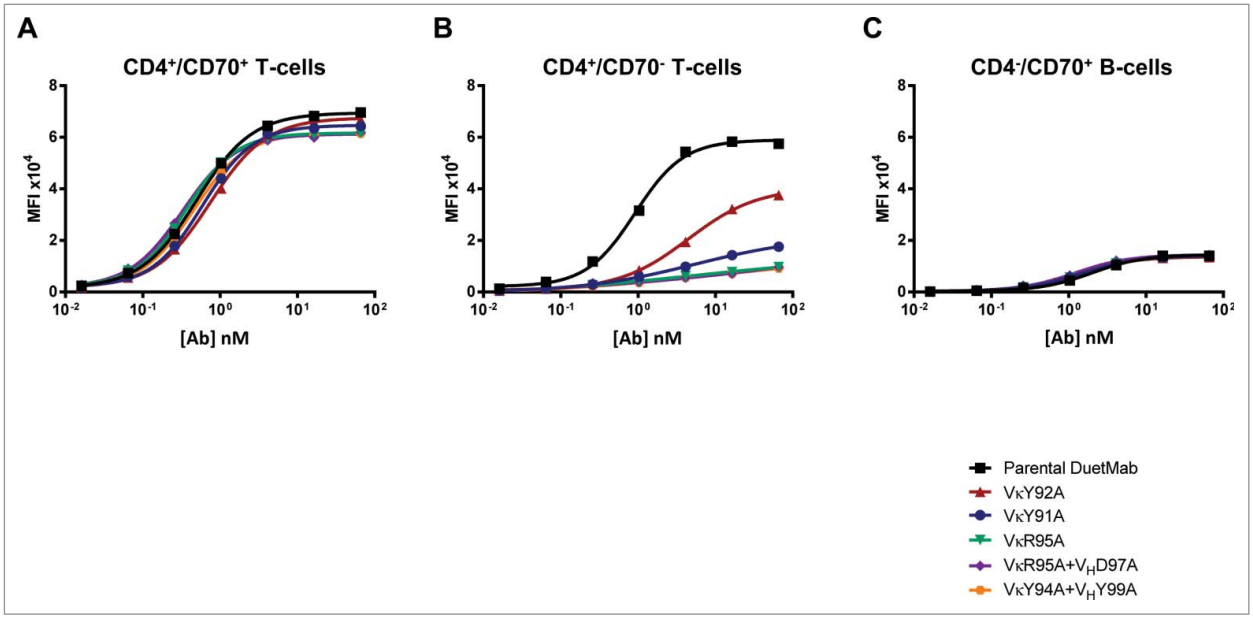

Figure 3. Cell binding of various DuetMabs variants. Binding of anti-CD4/CD70 DuetMab variants to (**A**) $CD4^+/CD70^+$, (**B**) $CD4^-/CD70^+$ and (**C**) $CD4^+/CD70^-$ lymphocytes in a mixture of all 3 cell types. All variants with reduced affinity to CD4 exhibited improved binding selectivity over the parental DuetMab whereas their binding to target $CD4^+/CD70^+$ T cells was not substantially impaired. Each point represents the mean values of triplicate wells and the standard deviation is represented by error bars.

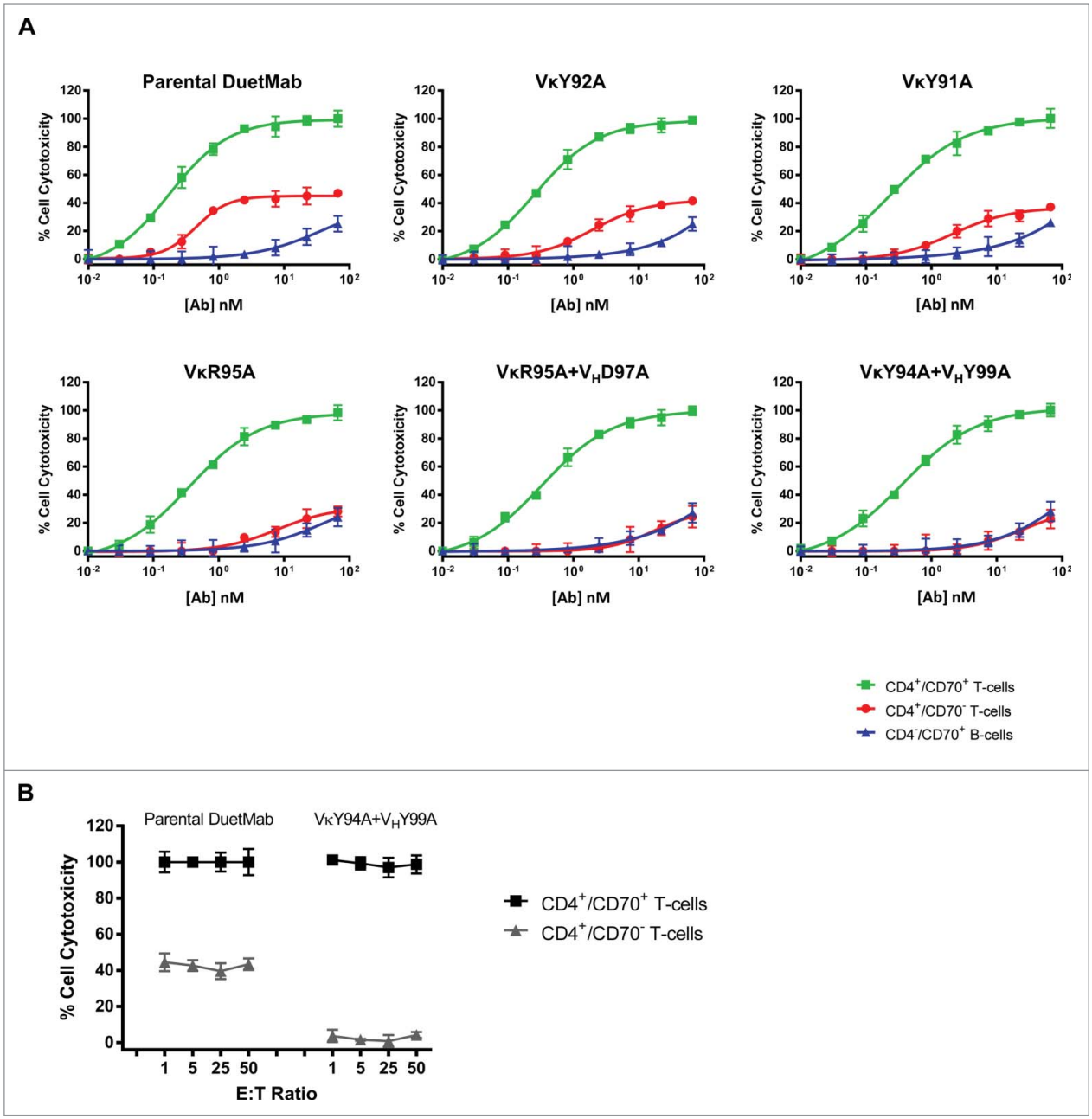

Figure 4. ADCC activity of various DuetMabs variants. (**A**) Selective ADCC depletion of $CD4^+/CD70^+$ T cells in a cell-mixture also containing non-target $CD4^+/CD70^-$ T cells and $CD4^-/CD70^+$ B cells. (**B**) ADCC activity of parental and anti-CD4 VκY94A+V_HY99A/CD70 DuetMabs against individual populations of $CD4^+/CD70^+$ and $CD4^+/CD70^-$ T-lymphocytes at varying E:T ratios. Each point in these studies represents the mean values of triplicate wells and the standard deviation is represented by error bars.

Table 3. Cytotoxicity of various CD4 affinity-reduced DuetMab variants

	Concentration (nM) needed for 20% target cytotoxicity					
Cell	**Parental DuetMab**	**VκY92A**	**VκY91A**	**VκR95A**	**VκR95A+V_HD97A**	**VκY94A+V_HY99A**
$CD4^+/CD70^-$ T cells	0.5	2	4	21	57	62
$CD4^+/CD70^+$ T cells	0.06	0.07	0.07	0.09	0.07	0.08

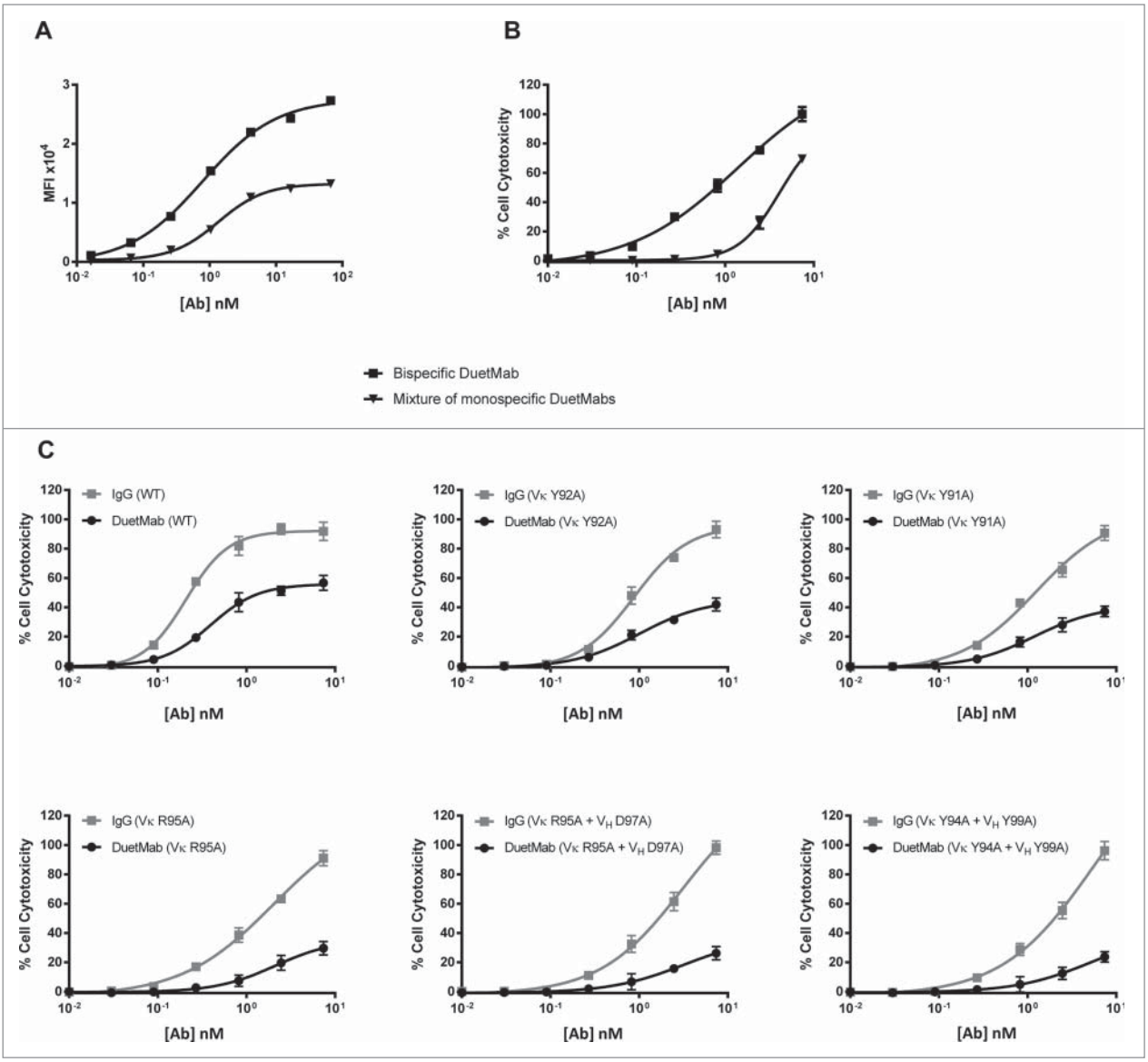

Figure 5. Effect of antibody valence on cell binding and ADCC activity. (**A**) Cell binding and (**B**) ADCC activity of anti-CD4 VκY94A+V_HY99A/CD70 and 2 monospecific (anti-CD4 VκY94A+V_HY99A/NMGC and anti-CD70/NMGC) DuetMabs at equimolar concentration against $CD4^+/CD70^+$ T cells alone. (**C**) Non-target ADCC activity of anti-CD4 variants formatted as either monovalent anti-CD4/CD70 DuetMab or bivalent anti-CD4 IgG against $CD4^+/CD70^-$ T cells alone. Each point in these studies represents the mean values of triplicate wells and the standard deviation is represented by error bars.

without impairing the overall activity of the molecule; and 5) an increase in antibody valence and avidity have a significant detrimental effect on the ability to promote target selectivity.

Improving target specificity should result in better therapeutic efficacy. This is particularly meaningful in cancer as most tumor-associated-antigens used in targeted therapy are also expressed on normal peripheral cells and tissues, albeit at disparate density.[11,24,25] We propose that the factors we have identified represent key parameters and should be taken into consideration when designing bsAbs as protein therapeutics. Future studies will determine the in vivo relevance of these observations.

Materials and Methods

Data

All experiments described in this manuscript are representative of at least 2 independent measurements.

Cells

Human $CD4^+$ T lymphocytes were obtained from peripheral blood mononuclear cells of healthy donors, using magnetic bead separation (Stemcell technologies) as per the manufacturer's instructions. Freshly isolated $CD4^+$ T cells were stimulated for 4 days with plate-bound anti-CD3/anti-CD28 mAbs in the

presence of either interleukin (IL)-12 (20 ng/ml) and IL-2 (20 ng/ml) for $CD4^+/CD70^-$ expression or IL-1 (20 ng/ml) and transforming growth factor β (5 ng/ml) for $CD4^+/CD70^+$ expression. All antibodies and cytokines were purchased from R&D Systems. T1B-196 $CD4^-/CD70^+$ B cells were obtained from the American Type Culture Collection and cultured in RPMI-1640 supplemented with 15% heat-inactivated (HI) fetal bovine serum (FBS). Human KC1333 NK cell line expressing human FcγRIIIA and FcεRIγ was obtained from BioWa Potelligent Technology and cultured in Advanced RPMI 1640 supplemented with 10% HI FBS, 200 μg/ml geneticin, 4 mM glutamine and 4.65×10^5 IU/ml IL2. The NK92/NFAT cell line is an in-house cell line engineered to stably express the high-affinity FcγRIIIa-V158 receptor and a luciferase reporter gene driven by the NFAT promoter. These cells were cultured in RPMI-1640 with glutamax supplemented with 12.5% HI FBS, 12.5% HI horse serum, 2 mM glutamine, 500 μg/ml geneticin, 100 μM 2-mercaptoethanol and 3.72×10^3 IU/ml IL2.

Mutagenesis and production of anti-CD4 antibodies

Alanine mutagenesis of targeted residues in CDRH3 and L3 of the anti-CD4 ibalizumab mAb was done by site-directed mutagenesis using standard PCR techniques. The mutated VH and VL domains were then cloned into an Orip/EBNA-1-based episomal mammalian expression plasmid, pOE.[26] DuetMab antibodies were cloned and produced essentially as described.[20] Briefly, antibodies were produced by transient transfection of HEK293F cells using 293fectin™ (Invitrogen) in serum-free Freestyle™ medium (Invitrogen) according to the supplier's recommended procedures. Cell culture supernatants were harvested 6 days after transfection and filtered through a 0.22 μm sterile filter. Concentration of antibodies in cell-culture supernatants was measured using an Octet384 instrument (ForteBio) according to the manufacturer's protocol. Antibodies were purified by protein A affinity chromatography using MabSelect SuRe resin (GE Healthcare) and subsequently buffer-exchanged in phosphate buffered saline (PBS) pH 7.2. The concentration of purified antibodies was determined by their absorbance at 280 nm. Purity and oligomeric state was assessed using a BioAnalyzer (Agilent) and size exclusion chromatography (SEC). The expected mass of DuetMab molecules was confirmed by RP-HPLC and ESI-MS as previously described.[20]

Binding kinetics measurements

IgG and DuetMab binding affinity to soluble monomeric forms of CD4 (R&D Systems, Cat. # 514-CD-050/CF) and CD70 (Origene, Cat. # TP300410) was measured using an Octet384 instrument (ForteBio). For kinetic analysis of the IgGs, purified antibodies at 10 μg/ml in PBS pH 7.2, 3 mg/ml BSA, 0.05% (v/v) tween 20 (assay buffer) were captured on anti-human IgG Fc biosensors (ForteBio). The loaded biosensors were washed with assay buffer to remove any unbound protein before carrying out association and dissociation measurements with serial dilutions of CD4 or CD70. For kinetic analysis of DuetMab molecules, streptavidin biosensors (ForteBio) were used to capture biotinylated CD4 or CD70 at 5 μg/ml in assay buffer. Following a washing step, association and dissociation measurements were carried out using serial dilutions of purified DuetMab. Kinetic parameters (k_{on} and k_{off}) and dissociation constant (K_D) were calculated from a non-linear fit of the data using the Octet384 software v.7.2.

Receptor density analysis

Receptor density studies were performed by flow cytometry on a MACSQuant VYB (Miltenyl Biotec). Purified parental anti-CD4 (ibalizumab) and anti-CD70 (2H5) IgGs were first labeled with Alexa Fluor 647 labeling kit (Invitrogen) according to the manufacturer's instructions. Antibody concentration and fluorochrome to protein (F:P) ratio was calculated using a ND-1000 spectrophotomer (NanoDrop). $\sim 4 \times 10^6$ cells/ml were washed twice with ice-cold FACS Buffer (PBS pH 7.2, 2% FBS, 2 mM EDTA and 0.1% sodium azide) and incubated with saturating (>= 20 μg/ml) concentration of conjugated antibodies for 30 min at 4°C. After 2 washes with FACS buffer, cells were fixed in ice-cold 1.8% paraformaldehyde (PFA). Detection of bound antibodies was performed on MACSQuant VYB using MACSQuantify™ software and results were analyzed with the FlowJo program (Tree Star). For quantitation of CD4 and CD70 density on cells, Quantum Alexa Fluor 647 MESF (Molecules of Equivalent Soluble Fluorochrome) beads (Bangs Laboratories) were analyzed on the flow cytometer using similar settings and a standard curve was established. The MESF calculated from the QuickCal software was used was then divided by the antibody F:P ratio to give corrected ABC (Antibody Binding Capacity).

IgG cell binding assays

IgG cell binding studies were performed by flow cytometry using a LSR II (Becton Dickinson) instrument. $\sim 5 \times 10^4$ $CD4^+/CD70^-$ cells/well were used in each experiment. Cells were washed twice with FACS buffer and incubated with serial dilutions of the tested antibodies for 1 h at 4°C. After washing twice with FACS buffer, FITC-conjugated goat anti-human Fc_γ (Jackson ImmunoResearch) was added for 45 min at 4°C. Data was analyzed with the FlowJo analysis software and antibody binding determined by mean fluorescence intensity (MFI).

DuetMab cell binding assays

Binding of DuetMab molecules was assessed using $CD4^+/CD70^-$, $CD4^+/CD70^+$ and $CD4^-/CD70^+$ cells alone or combined into a single preparation prior to staining. To enable subsequent identification by flow cytometry, each population was first stained with CellTrace™ Violet, CSFE or CellTrace™ Far Red (Life Technologies, NY), respectively, and combined (when applicable) at 1:1:1 ratio in FACS buffer at a final concentration of $\sim 9 \times 10^5$ cells/ml ($\sim 3 \times 10^5$ cells/ml of each population). $\sim 9 \times 10^4$ cells/well were then incubated with serial dilutions of DuetMab antibodies for 1 h at 4°C. After washing twice with FACS buffer, cell-bound antibody was detected with PE-conjugated anti-human Fc_γ (Jackson ImmunoResearch). Flow cytometry was performed on an LSR Fortessa (Becton Dickinson), with individual cells populations gated based on tracer dye expression and doublets excluded by physical scatter properties. Data was

analyzed with the FlowJo analysis software and DuetMab binding determined by mean fluorescence intensity (MFI).

Antibody-dependent cell-mediated cytotoxicity assays

To measure DuetMab variants selective ADCC against $CD4^+/CD70^-$, $CD4^+/CD70^+$ and $CD4^-/CD70^+$ cells, we used a flow-based cell enumeration method. More particularly, after staining each population with a tracer dye as described in the previous section, cells were combined at a 1:1:1 ratio in RPMI 1640 with glutamax supplemented with 10% FBS and incubated with KC1333 NK effector cells at an effector:target (E:T) ratio of 2.5:1. DuetMabs at various concentrations were added and cells were incubated for 6 h at 37°C in 5% CO_2. Cells were then stained with propidium iodide (for viability) and PE-Cy7 CD16 (for separation of KC1333 effectors) and analyzed by flow cytometry on an LSR Fortessa. To enumerate each cell population, data was processed using the FlowJo analysis software and after exclusion based on physical scatter properties and PI stain, live cells were separated by tracer dye and counted within a defined time gate. To adjust for any discrepancy in flow rate, counts were normalized against KC1333 cells as their numbers and viability were unaffected by ADCC activity. They also provided a more consistent reference than beads, which would frequently stick to dead cells. % cell cytotoxicity was calculated based on the change in cell number relative to a no-antibody control and normalized against parental DuetMab killing of $CD4^+/CD70^+$ target cells.

Whenever a single cell population was examined, we used a reporter assay that relied on a bioluminescent marker to quantify functional ADCC. Compared with the enumeration-based method described above, essentially similar results were obtained (data not shown). The ADCC assay used here is a modified version of a previously reported ADCC Reporter Bioassay.[27] Here, ADCC activity was extrapolated from the binding mediated by the effector NK92/NFAT cells to cell-bound antibody. The human NK92/NFAT cell line was engineered to express the high-affinity FcγRIIIa-V158 receptor and a luciferase reporter gene driven by the nuclear factor of activated T cells (NFAT) promoter. ADCC activity was quantified through the luciferase produced as a result of NFAT pathway activation and measured by the Steady-Glo Luciferase Assay system (Promega). $CD4^+/CD70^+$ or $CD4^+/CD70^-$ cells were seeded in 96-well plates at a density of $\sim 1 \times 10^4$ cells/well in RPMI-1640 with glutamax and supplemented with 12.5% HI FBS, 12.5% HI horse serum, 2 mM glutamine, 500 μg/ml geneticin, and 100 μM 2-mercaptoethanol. NK92/NFAT cells were mixed with target cells at different effector:target (E:T) ratio varying from 1:1 to 50:1. DuetMabs or IgGs at various concentrations were added and the cells incubated for 5 h at 37°C in 5% CO_2. Cells were then exposed to Steady-Glo luciferase substrate for ~ 50 min and OD_{409} was measured using an EnVision 2104 Multilabel plate reader (PerkinElmer).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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