ENHANCING ANTIBODY Fc HETERODIMER FORMATION THROUGH ELECTROSTATIC STEERING EFFECTS: APPLICATIONS TO BISPECIFIC MOLECULES AND MONOVALENT IgG

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Running Title: Design of Fc Heterodimer and Applications

Naturally occurring IgG antibodies are bivalent and monospecific. Bispecific antibodies having binding specificities for two different antigens can be produced using recombinant technologies and are projected to have broad clinical applications. However, co-expression of multiple light and heavy chains often leads to contaminants and poses purification challenges. In this work, we have modified the CH3 domain interface of the antibody Fc region with selected mutations so that the engineered Fc proteins preferentially form heterodimers. These novel mutations create altered charge polarity across the Fc dimer interface such that co-expression of electrostatically matched Fc chains support favorable attractive interactions thereby promoting desired Fc heterodimer formation while unfavorable repulsive charge interactions suppress unwanted Fc homodimer formation. This new Fc heterodimer format was used to produce bispecific single-chain antibody fusions and monovalent IgGs with minimal homodimers contaminants. The strategy proposed here demonstrates the feasibility of robust production of novel Fc-based heterodimeric molecules and hence broadens the scope of bispecific molecules for therapeutic applications.

Monoclonal antibodies have become an increasingly important class of therapeutic molecules for numerous indications including cancer, inflammatory diseases and viral infections(1). By specifically binding to their targets such as cytokines in circulation or receptors on the cell surface, antibodies can either block or activate certain biochemical pathways. In addition, antibodies can also recruit other effector cells from the immune system for the selective destruction of cells expressing their target antigens. Modifications of the antibody IgG format differing in valence level or the degree of specificity or both, through protein engineering, offer the promise of extending the useful therapeutic properties of antibodies even further(2).

In IgG format antibodies, the two antigen binding fragment (Fab) arms are connected by a flexible hinge region to the homodimeric Fc fragment. The Fc region of IgG mediates antibody effector functions by its interactions with Fc receptors on effector cells and confers a long serum half life in vivo through its interactions with neonatal Fc receptors(3). In addition, Fc can be easily produced in large quantities from mammalian cells and other hosts. For these reasons, the Fc fragment is commonly used as a partner to make fusions with other therapeutic proteins for in vivo applications(4). The Fc is often capable of
bringing antibody like qualities to the fusion protein. Due to the inherent dimeric nature of the Fc fragment, Fc fusions are produced as bivalent homodimeric proteins. However, for certain applications such as the production of bispecific antibodies where heterodimeric assembly between two different molecules is required, it is desirable to have Fc fragments that can efficiently heterodimerize. While simple co-expression of two different Fc heavy chains can lead to the formation of some heterodimer, the resulting products also contain significant amounts of homodimers that need to be purified away (5). Methods that can reliably promote Fc heterodimer formation while efficiently suppressing the homodimer forms are thus required to facilitate scalable and efficient productions to support clinical trials and commercial marketing of bi-specific protein-based therapies.

A strategy was proposed earlier by Carter and co-workers to produce Fc heterodimer using a set of “knob-into-hole” mutations in the CH3 domain of Fc (5-8). These mutations lead to the alteration of residue packing complementarity between the CH3 domain interface within the structurally conserved hydrophobic core so that the formation of the heterodimer is favored compared to homodimers. Although the strategy led to higher heterodimer yield, the homodimers were not completely suppressed (7). In this work, we explored the feasibility of retaining the hydrophobic core integrity while driving the formation of Fc heterodimer by changing the charge complementarity at the CH3 domain interface. Taking advantage of the electrostatic steering mechanism, we were able to efficiently promote Fc heterodimer formation with minimum contamination of homodimers through mutation of two pairs of peripherally located charged residues. In contrast to the knob-into-hole design, the homodimers were evenly suppressed due to the nature of the electrostatic repulsive mechanism. This new forms of Fc heterodimer greatly broadens our options for making asymmetrical Fc fusion proteins, where each Fc chain is fused with a different function group such as antibody fragment, peptide, soluble receptors, etc.

**EXPERIMENTAL PROCEDURES**

**Computational Analyses**

The computational scheme used to analyze the CH3 domain interface and to identify the charged residue pair that is likely to significantly impact dimer formation is shown in Supplementary Figure 1. A total of 27 (46 chains; some were deposited as dimer while others as monomer) antibody crystal structures which had co-ordinates corresponding to the Fc region were identified from the Protein Data Bank (PDB) (9) using a structure based search algorithm (10). Examination of the identified Fc crystal structures revealed that the structure determined at highest resolution corresponds to the Fc fragment of Rituximab bound to a minimized version of the B-domain from protein A called Z34C (PDB code: 1L6X)(11). The biological Fc homodimer structure for 1L6X was generated using the deposited Fc monomer co-ordinates and crystal symmetry. Two methods were used to identify the residues involved in the CH3-CH3 domain interaction: (i) contact as determined by distance limit criterion and (ii) solvent accessible surface area analysis. According to the contact based method, interface residues are defined as residues whose side chain heavy atoms are positioned closer than a specified limit (4.5Å) from the heavy atoms of any residues in the second chain. The second method involves calculating solvent accessible surface area (ASA) of the CH3 domain residues in the presence and absence of the second chain (12). The residues that show difference (>1Å²) in ASA between the two calculations are identified as interface residues. Both the methods identified similar set of interface residues. Further, they were consistent with the published work (13). Supplementary Table 1 lists the twenty four interface residues identified based on the contact criterion method, using the distance limit of 4.5Å. These residues were further examined for structural conservation. For this purpose, 46 Fc crystal structures identified from the PDB were superimposed and analyzed by calculating root mean square deviation for the side chain heavy atoms. The CH3 domain interface buried (% ASA ≤ 10; % ASA refers to ratio of observed ASA to the standard ASA of amino acids (12)) and exposed (% ASA > 10)
residue positions were identified using the solvent accessible surface area calculation.

**Generation of Heterodimer Constructs Using Fc Charge Pair Mutation**

A rat anti-mouse NKG2D antibody, designated M315, was generated through conventional hybridoma fusions and the DNA sequences encoding the variable heavy chain (VH) and variable light chain (VL) were used to construct M315scFv-Fc using previously described method(14). The M315 scFv-Fc and hulgG1Fc were cloned into the pTT5 mammalian expression vector and the two constructs were used to co-transfect 293 cells to assess the formation Fc/scFv-Fc heterodimer relative to Fc homodimer and scFv-Fc homodimer. The charge residue pairs in the CH3 region identified through computational analysis were changed to amino acid of opposite charge polarity on either human IgG1 Fc (dummy) or M315 scFv-Fc constructs. The mutations were generated using the QuikChange® mutagenesis kit from Stratagene and verified by DNA sequencing. The mutations are denoted by wild type residues followed by the position using the Eu numbering system and then the replacement residue in single letter code. The Fc sequence used in these two constructs was derived from human IgG1 non-(a) allotype, which has a Glu at position 356 and a Met at position 358. The CH3 sequences from the crystal structure are from a different IgG1 allotype, which has an Asp at position 356 and a Leu at position 368.

14D2 hamster anti-mouse p55TNFR antibody was obtained from Hiko Kohno. The variable heavy(VH) and variable light(VL) sequences were cloned from total RNA obtained from the hybridoma cells using degenerate primers to the 5’ sequence determined from Edman N-terminal sequence of purified 14D2 antibody and 3’ primers complimentary to sequence in the Constant Heavy 1(CH1) of hamster IgG1. The 14D2 Vh-hu light chain \( \kappa \) fusion was made by amplifying the 14D2 Vh with the following primers:

\[
\begin{align*}
5’ &\text{CTGGTGCTAGCGATATAGTGATGTCGC}\ &\text{and} \\
5’ &\text{CAGCCACCGTAGTTGATTTCCAGCTT}
\end{align*}
\]

GG. The amplicon was subcloned into a vector containing the sequence of the hu \( \kappa \) light chain constant sequence. The 14D2Vh-hu \( \kappa \)LC-hulgG1Fc fusion was spliced together by overlap extension using the following primers:

\[
\begin{align*}
5’ &\text{CGTTTAAACGTGACGGTTAAAACGCGCC} \\
5’ &\text{GGCATGTGTGAGTTTGATTTCCAGCTT} \\
5’ &\text{CAACAGGGGAGAGTGTGACAAAAACTC} \\
5’ &\text{GTGTTAACAGATCCGCCCGCGCTCTAG} \\
\end{align*}
\]

C CCC. The 14D2Vh-hulgG1-His\( \kappa \) fusion was constructed by amplification of the 14D2 Vh with the following primers to add AscI and NheI restriction sites 5’ and 3’ respectively, to ligate the 14D2Vh directly upstream to the hulgG1-His\( \kappa \) constant sequence in a mammalian expression vector.

To reduce the formation of light chain–Fc homodimer, charge mutations K409E:K392D were introduced on the Fc attached to the light chain and D399R:E356K were introduced on the Fc of the heavy chain. The heavy chain and light chain constructs were cotransfected into CHO cells to generate a stable pool. A similar set of constructs were also generated for 14D2 using mouse Fc backbone in which N399K:E356K (equivalent to D399K:E356K in human Fc) mutations were introduced on the heavy chain mouse IgG1 Fc and K409D:K392D (equivalent to K409D:K392D in human Fc) mutations were introduced on the light chain mouse IgG1 Fc fusion.

Bispecific scFv-Fc constructs were also generated using the Fc charge mutations. scFv constructs were first generated using the variable heavy and light chain sequences of OKT3 and anti- TARTK IgG1. The scFv fragments were cloned into a mammalian expression vector to generate scFv-Fc expression constructs(14). D399K:E356K mutations were introduced on the Fc backbone of OKT3 scFv-Fc vector. K409D:K392D mutations were introduced on the TARTK scFv-Fc construct. The two vectors were cotransfected to 293 cells and the scFv-Fc was purified from culture media using Mab Select Sure column and SEC then subjected to Mass Spec analysis.
**Protein Production and Analysis**

DNA was transfected into human embryonic kidney cell line 293 using Lipofectamine™ 2000 reagent (Invitrogen) or Polyethylenimine (PEI) depending on volume used. The cell culture supernatant was harvested 5-6 days after transfection and run on SDS-PAGE Gels under non-reduced conditions. The gel was then transferred to nitrocellulose membrane and subject to western analysis using peroxidase-conjugated goat anti-human IgG, antibody (Jackson ImmunoResearch Laboratories).

**Mass Spec Analysis of Bispecific scFv-Fc and Monovalent IgG**

The bispecific scFv-Fc and monovalent IgG samples were deglycosylated and followed by LC/MS analysis. About 20 µg of bispecific scFv-Fc was deglycosylated by incubation with 1 µl of PNGase F (New England) at 37°C overnight in its native solution. After deglycosylation, the bispecific scFv-Fc was analyzed by HPLC-ESI-TOF (Agilent 6210 TOF mass spectrometer in combination with an Agilent 1200 LC system, Santa Clara, CA). A Varian Pursuit 5µ Diphenyl 150 x 2.1 mm column was connected to the LC system and operated at 400 µL/min. The column temperature was 75°C, solvent A was 0.1 % TFA in water, and solvent B was 0.1 % TFA in acetonitrile. The gradient started at 25% B and increased to 80% B over 55 min. The TOF mass spectrometer was tuned and calibrated in the range of 100 to 4500 m/z. It has better than 3 ppm mass accuracy through continuous and automated reference mass introduction.

**T-cell Dependent Cytotoxicity Assay (TDC)**

U87 or U87TARTK cells (target cells) were seeded in full growth media at 10,000 cells/well (100uL) in clear bottom black well 96-well assay plates (Costar 9927) leaving one row empty for effector cell control. The cells were incubated at 37°C/5%CO₂ overnight. Percent cytotoxicity was calculated from the relative light units according to this formula: % cytotoxicity = ((total lysis) – (total spontaneous release))/((experimental release) – (spontaneous release)).

**Cell Culture & Peripheral Blood Mononuclear Cells**

U87 and U87-TARTK cells were cultured in DMEM supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 1mM Sodium Pyruvate and 0.1mM MEM non-essential amino acids. Cultures were split every 3 to 4 days at 1:5 and 1:10 ratio. Peripheral blood mononuclear cell enriched Leukopacks were obtained from Biological Specialty Corporation (Colmar, PA) and frozen at 1 x 10⁹/mL in 90% FBS/10% DMSO prior to use.

**TNF Mediated L929 Cytotoxicity Assay**

Murine fibroblast L929 cells (ATCC CCL-1) were maintained in Eagle’s minimal essential
medium (EMEM) supplemented with 10% Horse Serum and 1% PSG, 1% NEAA, 1% Sodium Pyruvate, and 1% HEPES, and maintained at 37°C with 5% CO2 in a humidified incubator. For the cytotoxicity assay, cells were plated in 96-well plates at 70,000 cells/well in 100uL media supplemented with Actinomycin-D (2ug/mL, Sigma Cat#A9415) and allowed to incubate for 2hrs. The indicated amounts of antibodies (0.4 – 50 ug/ml) were added to the wells and plates were incubated 18hrs. Following a wash with phosphate-buffered saline (PBS), alamar blue (Invitrogen DAL1025) was added at 1:10 (v/v) dilution in complete EMEM and cells were incubated an additional 4hrs. Plates were read on a fluorescence plate reader (excitation 544 nm and emission 595 nm). To assess antagonism of TNF signaling, antibodies were preincubated with the cells at the indicated concentrations (1 uM to 50 pM) for 30 minutes, after which the cells were stimulated with 5pM muTNF (R&D Systems Cat#410-MT) for 18 hrs. Cellular viability was assessed with Alamar Blue as described for the agonist assay above. Percent inhibition of cell death was calculated using medium-stimulated and TNF-stimulated control wells.

Pharmacokinetics study
Experimental cohorts of C57BL/6 mice were intraperitoneally administered a single 100ug dose of various anti-murine p55 constructs. At multiple time points following injection, animals were euthanized and blood was collected for analysis. Serum Antibody Concentrations and PK Analysis: Serum concentrations of various 14D2 constructs were determined using a sandwich ELISA method. Recombinant murine sTNFRI (murine p55; R & D Systems) and horseradish-peroxidase conjugated goat anti-murine IgG Fc fragment specific polyclonal sera (Pierce) were used as the capture and the detection antibody, respectively. Plate wells were developed using tetramethyl-benzidine and peroxide substrate solution (Kirkegaard & Perry). The resulting colorimetric reaction was quenched with 10% phosphoric acid (VWR) and optical densities were determined at a wavelength of 450-650nm.

The conversion of OD values into concentrations for the quality control samples and serum specimens was achieved through Watson LIMSTM software mediated comparison to a concurrently analyzed standard curve, which was regressed according to a four-parameter logistic model. Non-compartmental pharmacokinetic analyses, including half-life determinations, were performed on mean concentration-time data using Watson. Area under the curve values were calculated using the log-linear trapezoidal method.

Mouse xenograft studies
Seven week old NOD.SCID female mice were obtained from Harlan laboratories and maintained under sterile conditions. Animals were provided food (Teklab 2920) and acidified water ad libitum. All animal care complied with Canadian Council on Animal Care guidelines. U87/TARTK tumor cells were mixed with purified T cells (Biological Specialty Corporation 215-01-10) in serum free RPMI-1640 and mixed 1:1 with matrigel (BD Biosciences 356237). The final concentration was 20x10⁶ cells/mL tumor and 25x10⁶ cells/mL T cells. Animals were implanted with 0.1mL subcutaneously in right flank of mice on day 0. Treatments with anti-CD3/TARTK bispecific scFv-Fc (14 or 140 ug) were administered in 200 uL DPBS intravenously via the tail vein. Initial treatments were given 3 hours after tumor implant and a second treatment was administered 4 days later. Tumors were measured twice weekly and tumor volume was calculated using the formula (width² x length)/2. Mean tumor volumes and standard error for each measurement data were determined and used to generate the graphs.

RESULTS

Enhancing antibody Fc heterodimer formation through electrostatic steering effects
Crystal structures of the Fc region of the antibodies show extensive protein-protein interactions (surface area buried = 2469Å²) occurring between the CH3 domains (13,15) and the high affinity (Kd=10 pM) CH3-CH3 interaction is the primary driver for Fc dimerization (Figure 1a) (16). A total of 27 (46
chains) antibody crystal structures with coordinates corresponding to the Fc region were identified in the Protein Data Bank (PDB) (9) using a structure based search algorithm (10). The structure determined at highest resolution (PDB code: 1L6X(11), 1.6Å) was used to identify the CH3-CH3 domain interface residues (24 residues; Supplementary Table 1). These residues were further examined for structure and sequence conservation (Supplementary Figure 1). For this purpose, the identified 46 Fc crystal structures were superimposed and analyzed by calculating the root-mean-square-deviation for the side chain heavy atoms. The analysis revealed the CH3 domain interface includes a central hydrophobic core region surrounded by charged residues that interact to promote dimer formation through favorable electrostatic interactions around the rim of the interface (Figure 1b; Supplementary Figure 2). High structural conservation for the hydrophobic residues at the core of the interface as well as for a few of the charged residues was noted. Further examination of the residues involved in the CH3-CH3 domain interactions (Supplementary Table 1) revealed four pairs of oppositely charged residues along the interface (Asp356---Lys409′, Glu357---Lys370′, Lys392---Asp399′, & Asp399---Lys409). Comparison of human and mouse IgG subclasses also reveals high conservation for these charged residues (Supplementary Figure 3). We hypothesized that the overall charge polarity could be altered through mutagenesis of specific residues such that attractive electrostatic interactions would favor heterodimer formation while repulsive charged interactions would disfavor homodimer formation. In addition, exploiting charged residues as opposed to hydrophobic residues at the CH3 domain interface may have benefits in terms of retaining the generally favorable biophysical properties of the Fc. It has long been established that the hydrophobic core of protein domains plays an important role in protein folding and stability(17).

Examination of the four charged residue pairs reveals that in the case of the Lys409---Asp399′ pair both the residues are structurally conserved and buried (Table 1). Studies have suggested that structurally conserved and buried residues contribute significantly to protein-protein association (18,19). Therefore, the Lys409---Asp399′ pair was chosen for a proof-of-concept experimental study. It must also be noted here that due to the 2-fold symmetry present in the CH3-CH3 domain interaction, each unique interaction will be represented twice in the structure (for example, Asp399---Lys409′ & Lys409---Asp399′; Figure 1c). The strategy to enhance heterodimer formation using the charged residue pair is schematically shown in Figure 1d. In the wild type Fc, the Lys409---Asp399′ (K409---D399′) interaction favors both heterodimer and homodimer formation. A double mutation switching the charged residue polarity (K409D in first chain and D399′K in the second chain) leads to unfavorable repulsive interactions for the formation of homodimer while preserving favorable interaction for the heterodimer formation. The strong repulsive interactions (K409D --- D399′K & K409′---D399′K) between the charged residues may hinder homodimer formation in the kinetic pathway of dimer assembly.

In order to assess relative yields of heterodimer and homodimer, a model system was used in which two Fc fusions with significant size difference, a scFv-Fc fusion and a Fc fragment (Figure 2a), were co-expressed in 293 cells. The secretion of the three possible dimeric products (scFv-Fc/scFv-Fc and Fc/Fc homodimers, and scFv-Fc/Fc heterodimer), could be assessed on a SDS-PAGE under non-reduced condition. When the two Fc fusion constructs were co-expressed, less than 50% of the product was scFv-Fc/Fc heterodimer. The introduction of a single charge pair alteration (K409D on the Fc fragment and D399′K on the scFv-Fc) significantly increased the ratio of scFv-Fc/Fc heterodimer relative to both scFv-Fc and Fc homodimers (Figure 2b). Similar enhancement of heterodimer formation was also observed for other similar charge mutant combinations such as K409D/D399′R, K409E/D399′K and K409E/D399′R (Figure 2b), underscoring the importance of charge polarity complementation for the formation of Fc heterodimer.

When additional charge pair mutations were combined with K409D/D399′K mutation, a
further increase of heterodimer formation and decrease of homodimers was observed. The combination of K409D:K392D in the Fc fragment with D399’K:E356’K in the scFv-Fc resulted in heterodimer formation almost exclusively. Other combinations such as K409D:K392D/D399’K:D357’K and K409D:K370D/D399’K:D357’K also offered significant improvement over the K409D/D399’K variant alone (Figure 2c). Analogous constructs with the knobs-into-holes mutations (T366W/T366’S:L368’A:Y407’V) also formed primarily heterodimer, although a small amount of scFv-Fc homodimers could still be detected under the same experimental conditions (Figure 2c, Supplementary Figure 4a and Supplementary Table 2). It may be noted here that input DNA ratio can influence the relative proportions of the Fc dimer formation as emphasized in earlier work(7).

The effects of charge pair combinations on the formation of homodimers and heterodimer were further evaluated separately in a different experiment. As shown in Figure 2d, although both K409D:K392D and D339’K:E356’K double mutant can still form homodimers when expressed alone, they preferentially form heterodimer when expressed together. Interestingly, Fc molecules with three mutations wherein positive-charged residues were changed to negative-charged residues (K409D:K392D:K370D) or negative-charged residues were changed to positive-charged residues (D399K:E356K:E357K) could not be detected as homodimers. However, heterodimer could be produced when the two electrostatically matched triple mutant variants were co-expressed together, although the overall product yield was significantly reduced (Figure 2d). This observation suggests that charge complementation can critically influence Fc dimer formation. After a survey of many variants, we have determined that overall the combination of two pairs of charge mutations (K409D:K392D:D399’K:E356’K) on two respective Fc chains gave the best results for producing predominantly Fc heterodimers without a significant reduction of product yield.

Production of bispecific scFv-Fc using engineered Fc heterodimer

A bispecific molecule based on Fc heterodimer with charge pair mutations was constructed by fusing two scFv fragments with different binding specificities, one against human CD3 (OKT3)(20) and one against a tumor associated receptor tyrosine kinase (TARTK, unpublished), to the Fc heterodimer (Figure 3a). Co-transfection of mammalian cells with expression vectors encoding the two scFv-Fc fusion led to the secretion of single species production with a molecular weight of 120 KDa into the culture media (Figure 3a). The product was purified, deglycosylated, subjected to intact mass analysis by mass spectrometry. As shown in Figure 3b, the whole mass of the predominant fraction, representing more than 98% of product, matches the expect mass of the bispecific scFv-Fc heterodimer. The bispecific antibody was shown to be able to bind both CD3 on human T cells and TARTK expressed on cancer cells (Figure 3c). When incubated together with human PBMC and human glioma cell line U87 that has been transduced to stably express TARTK, the bispecific antibody can specifically induce the killing of U87/TARTK cells but not parental U87 cells at very low dose (Figure 3d). The killing activity is not due to the activity of conventional antibody dependent cellular cytotoxicity (ADCC) as the anti-TARTK human IgG1 failed to kill the U87/TARTK cells. A similar control bispecific antibody that binds to CD3 and an irrelevant target also failed to kill the U87/TARTK cells (Figure 3d). The CD3xTARTK bispecific antibody also showed dose dependent inhibition of tumor growth of U87-TARTK cell in xenograft model using NOD/SCID mice. Due to the presence of Fc region, the bispecific scFv-Fc fusion has a much longer in vivo half life in mouse and can be dosed infrequently than other types of CD3 bispecific scFv fragments. With two doses of 140 ug antibody (7mg/kg) i.v. on day 0 and day 4, the bispecific antibody completely suppressed the growth of TARTK positive glioma in a xenograft model where human T cells were implanted together with the tumor (Figure 3e).
Production of monovalent IgG using engineered Fc heterodimer

The charge pair based Fc heterodimer was also used to generate a novel form of monovalent IgG. As illustrated in Figure 4a, monovalent IgG is a product of a single Fab fusion to the heterodimer Fc. Compared to the bivalent four-chain structure of full IgG, the monovalent IgG is a two-chain molecule with only a single antigen-binding moiety. The heavy chain (HC) of this molecule is the same as in a standard IgG. The light chain (LC) is further extended by fusion with Fc using a modified hinge sequence. The resulting molecule should bind to antigen monovalently, similar to a Fab molecule, and should retain a pharmacokinetic profile similar to an IgG.

14D2 IgG is a potent antagonistic antibody to mouse TNF receptor 1 (TNFR1). It can efficiently block TNF mediated cytotoxic activity in L929 cells. However, it also activates TNFR1 at low doses in the absence of TNF. The agonistic activity of 14D2 IgG is due to the crosslinking of TNFR1 by the full IgG molecule, as the Fab fragment of 14D2 is free of agonistic activity (data not shown). To generate a pure antagonistic antibody with long serum half life for in vivo studies, a monovalent form of 14D2 IgG molecule was constructed. When the two transcripts were introduced into mammalian cells, only two of three possible products, HC/LC-Fc heterodimer and LC-Fc homodimer, were detected in the media (Supplementary Figure 5). The HC homodimer cannot be secreted due to the unpaired CH1 domain(21). When the wild type Fc sequence was used, only 50%-60% of the product was the desired monovalent IgG form with the rest consisting of LC-Fc homodimer. As a result, three times more LC-Fc was seen on SDS-PAGE gel than HC under reduced condition (Figure 4a). However, when charge pair mutations were used in the Fc constructs, the predominant product in the media was HC/LC-Fc heterodimer. As a result, the overall ratio of heavy chain to LC-Fc of the purified product on reduced SDS-PAGE gel is close to 1:1. This was confirmed by mass spec analysis. More than 90% of product after protein A purification was HC/LC-Fc heterodimer (data not shown). The final purified yield of the 14D2 monovalent IgG from a stable pool of CHO cells was approximately 100mg per liter.

The agonistic and antagonistic activity of the purified monovalent IgG was evaluated in L929 cytotoxicity assays. As shown in Figure 4b, monovalent IgG, either as human Fc or mouse Fc heterodimer, exhibited good potency in blocking TNF mediated cytotoxicity with an IC50 value similar to that of the bivalent IgG. However, the two monovalent IgG preparations were free of agonist activity. As shown in Figure 4c, the monovalent IgG did not lead to any decreased cell viability when incubated with L929 cells. This was in contrast to the 14D2 full IgG, which could induce killing of L929 cells in the absence of TNF. The agonistic activity of 14D2 was not detected for the Fab format, indicating its dependence on the bivalency of IgG. Importantly, both forms of the 14D2 monovalent IgG exhibited similar pharmacokinetic profiles as the full length IgG when injected in mice (Figure 4d), demonstrating that incorporation of the charge pair mutations did not compromise the ability of the Fc to mediate a long serum half life. Consistent with the in vitro analysis, 14D2 monovalent IgG could also block TNF mediated chemokine induction in vivo and demonstrated reduced agonist activity in vivo relative to the full length 14D2 IgG (data not shown). Overall, these observations suggest that monovalent IgG is capable of maintaining its stability and biological activity in vivo and the charge pair based Fc fusion proteins can serve as a general platform for making heterodimer-based proteins for in vivo applications.

DISCUSSION

In this work, we have demonstrated Fc heterodimers can be effectively produced by taking advantage of the electrostatic steering mechanism. Studies have shown that electrostatic steering effects can be exploited to alter the rate of association between proteins (22,23). For example, protein design and selection techniques have been successfully employed to produce novel heterodimeric coil-
coils (24-26). Although the design strategy in these studies included directed pairing of oppositely charged amino acid residues, further work supported the conclusion that formation of the most successful heterodimeric variants depends on interactions beyond electrostatics (27). In other studies, novel heterodimeric zinc finger nucleases have been engineered by introducing oppositely charged residues at the α-helical dimer interface thereby minimizing unwanted off-target DNA-cleavage due to homodimers (28,29). However, near complete suppression of homodimers also required altering hydrophobic interaction (28). These examples involve packing between two helices and the size of the protein-protein interfaces effected are much smaller than that of the ~2500Å² CH3 dimer interface. It is notable that, despite the high-affinity interaction between the two CH3 domains and the larger interface, only a few charged residue mutations can promote Fc heterodimer formation while effectively suppressing formation of homodimers. The study presented here suggests the electrostatic steering based mechanism can be used to engineer large protein-protein interface for heterotypic interactions.

In a pioneering study, Carter and co-workers engineered the antibody heavy chain for Fc heterodimerization using a “knobs-into-holes” strategy (5-8). The basis for creating knob and hole in the juxtaposed positions is that the knob and hole interaction will favor heterodimer formation, whereas the knob-knob and the hole-hole interactions will hinder homodimers formation due to the steric clash and deletion of favorable interactions, respectively. Although the knobs-into-holes strategy increased the production of heterodimer significantly, monomer and homodimer formations were not suppressed completely (6). The addition of inter-CH3 domain disulfide bond to the knobs-into-holes mutations further enhanced heterodimer formation up to 95% (7,30), approaching the selectivity achieved here in the charge pair strategy. The knob-into-hole mutations involve structurally conserved buried residues at the core of the interface. In contrast, the charge residues altered in this study are positioned around the rim of the interface. Unlike the knobs-into-holes method where a large amino acid is mutated to a smaller amino acid in the protein core (or vice versa for knob), the strategy involving charged residues is less likely to affect the packing of atoms at the hydrophobic core of the interface. For both strategies, a concern may arise that changing structurally conserved residues could lead to lower stability and affect other biophysical properties. Our qualitative comparison of differential scanning calorimetric profiles reveal similar melting temperature but higher enthalpy (area under the curve) for the charge pair design compared to the knobs-into-holes strategy (Supplementary Figure 4). While for both strategies the CH3 domain melting temperature (Tm) was decreased relative to WT, it still is well within a range that will support viable therapeutic protein productions as suggested by the PK analysis (Figure 4d). Free energy calculations using a computational method (EGAD (31)) also showed that the charge pair mutation has similar binding free energy (ΔΔGmut) compared to the knobs-into-holes mutations (Supplementary Table 3). A more detailed assessment of free energy comparison is complicated due to the lack of a folding model (e.g., two-state versus three-state) and multi-domain nature of the scFv-Fc.

Due to the two-fold symmetry present in the Fc crystal structure, each unique interaction at the CH3-CH3 domain interface is represented twice in the structure. The electrostatic steering mechanism proposed here exploits the same two-fold symmetry in order to effectively hinder the homodimer formation. Figure 1d shows how a single mutation (K409D in the first chain or D399'K in the second chain) makes use of the symmetry to impart a repulsive electrostatic interaction in the homodimer setting. This repulsive effect could be further enhanced when different charge mutations were combined, particularly when these mutations were spread to different contact region in the CH3 dimer interface (Supplementary Figure 6). In the extreme case where three charged residues were changed to oppositely charged amino acids, no Fc homodimers could be detected when the single construct was expressed alone (Figure 2d). In addition, the suppression of homodimers was equally effective for both “positively
charged” and “negatively charged” homodimers by the nature of electrostatic repulsive mechanism. This is in contrast to knob into hole design where knob-knob and hole-hole homodimers were not equally suppressed, due to the difference in shape complimentarity.

The ability to efficiently produce Fc heterodimers using the charge pair mutations potentially allows the generation of many different types of heterodimeric proteins by fusing various functional groups such as peptides, antibody fragments, protein ligands, or receptors to both ends of the Fc polypeptide chains. For example, a biologically active bispecific CD3 x TARTK antibody was produced in the form of a bispecific scFv-Fc fusion (Figure 3). Using this format, in principle any two antibodies can be combined into a single protein by making the appropriate scFv forms of the antibodies and fusing them to the heterodimeric Fc segments. In practice, the stability and expression levels of the individual scFv components have a large influence on the expression levels for the bi-specific molecules, in agreement with general observations regarding scFv expression(32). Conceptually, Fc heterodimers, could also be used to generate bispecific full IgGs. However, additional engineering work will be required to efficiently control the correct pairing the light chain (LC) with its heavy chain (HC) respectively. This issue could be circumvented by selecting a promiscuous LC that can be pair with both HCs and still allow binding, as suggested previously(7).

We also have demonstrated that homogenous production of monovalent antibodies (Fab-Fc fusions) can be facilitated by using the heterodimeric Fc format (Figure 4). This format is projected to be especially useful in situations where the goal is to antagonize a membrane-bound receptor (e.g. TNF receptor family molecules) without causing the activation of the receptor as conventional bivalent antibodies often do via cross-linking of the receptor. We show here that the monovalent form of anti-TNFR1 IgG based on the charge-pair Fc heterodimer strategy can be produced at high levels and antagonize the receptor without residual agonist activity (Figure 4). Compared to other forms of monovalent antibodies, such as Fab fragments, this Fab-Fc heterodimer fusion retains IgG-like long serum half life in vivo. This format provides a viable option for therapeutic applications where a monovalent antibody form with extended serum half-life is desired. A similar one-armed IgG construct using the knob and hole strategy was also developed for c-MET, a target to which a pure antagonist needs to be in the monovalent form(33).

In summary, we have demonstrated that the charged residues located around the rim of the CH3 domain interface can be engineered to promote Fc heterodimer formation. Using this engineered format, bispecific scFv-Fc as well as a monovalent IgG molecule having the desired in-vitro and in-vivo activities were effectively produced. Furthermore, pharmacokinetic (PK) analysis of the engineered human and mouse Fc heterodimers has shown that the serum half-life, which is an important property of the WT homodimer Fc, is retained in the designed molecules. The work presented here may have broad implications for engineering relatively large protein-protein interactions where electrostatic steering effects can be harnessed to effectively suppress unwanted homodimer forms while promoting the robust production of useful novel heterodimeric molecules.
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**FOOTNOTES**

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Author Contributions
K.G. conceived the concept, performed computational analysis, and wrote the manuscript. M.P. generated reagents and performed the proof of concept experiments. L.G. and M.S. purified and characterized protein samples. C.F. and A.W. generated reagents and performed experiments on monovalent IgGs. S.B.N. and T.B. performed experiments and interpreted data in the L929 cytotoxicity assay. M.R. was responsible for the pharmacokinetics studies. K.M. and I.F. developed and performed the T-cell dependent cytotoxicity assay. H.S. and I.F. were responsible for mouse xenograft studies. M.W. provided ideas and edited the manuscript. W.Y. designed experiments, analyzed data, and wrote the manuscript.
FIGURE LEGENDS

Figure 1. (a) Ribbon representation of an antibody Fc region crystal structure (PDB code: 1L6X) is shown here. Only the CH3 domain is involved in protein-protein interaction. (b) Figure shows the backbone trace of the CH3 domain interface structure with residues involved in the domain-domain interaction. The interface residues were identified using a distance cutoff (4.5Å) method. Structurally conserved and buried (solvent accessible surface area < 10%) residues are shown in the ball-and-stick model. Solvent exposed or structurally not conserved residues are shown in the stick representation. The analysis is based on the IgG1 crystal structure which is determined at high-resolution (1.65Å; 1L6X) 21. (c) Crystal structure of the CH3 domain homodimer with one domain shown in ribbon representation and the other domain shown in wire model. The Lys409 (Lys409' in the second domain) and Asp399 (Asp399' in the second) residues are shown in ball-and-stick model in order to illustrate each pair-wise interaction is represented twice in the structure. This is due to the two-fold symmetry present in the CH3-CH3 domain interaction. Schematics showing electrostatic interactions in the wild type (d) and in charge pair mutant (e) designed as an example to enhance heterodimer formation and hinder homodimer formation. In the case of WT, the charge-charge interaction favors both heterodimer and homodimer formation giving them equal probability. In the case of charge pair mutant, the charge-charge interaction favors heterodimer and disfavors homodimer formation.

Figure 2. (a) Schematic drawing of the two constructs used in the co-transfection experiments in 293 cells. The first construct encodes a scFv-Fc fusion. The second construct encodes a dummy Fc which does not have any domain attached to it. The products of the co-transfection experiments with different Fc variants were analyzed by SDS-PAGE under non-reduced condition and Western Blot as shown in (b), (c) and (d). (b) Effects of single charge pair mutations of the D399---K409' interaction pair on Fc heterodimerization. Products of either single transfection or cotransfections of Fc variants were shown. All four single charge pair variants (D399R/K409'E, D399K/K409'E, D399R/K409'D, and D399K/K409'D) led to higher yield for the scFv-Fc/Fc heterodimer (49% to 60%) compared to the homodimers (7% to 23%). The construct for the wild type Fc contained an extra tag of 33 amino acids, so its products appeared larger on SDS-PAGE. (c) Effects of charge pair mutation combinations on Fc heterodimerization. The K409D:K392D / D399'K:E356'K mutation combination and the WT/WT are highlighted. The T366W (knob) and T366'S:L368'A:Y407'V (hole) mutation combination was included as a control. (d) The effects of charge pair mutation combinations on Fc homodimer and heterodimer formations. The products of single transfection (homodimer formation) and cotransfection (homodimers and heterodimer) for each Fc variants were shown. The single transfection of the triple mutant constructs (K409D:K392D:K370D and D399K:E356K:E357K) did not yield any product of homodimer. However, the cotransfection of the two triple mutant constructs led a heterodimer formation.

Figure 3. (a) Schematic drawing of bispecific scFv-Fc generated by using the Fc heterodimer with charge pair mutations (left panel) and SDS-PAGE analysis (right panel) of the purified bispecific scFv-Fc heterodimer in non-reduced (lane 1) and reduced (lane 2) conditions. Anti-CD3 scFv OKT3 (VH1/VL1 in red) was fused to human Fc backbone with D399'K:E356'K mutations. Anti-TARTK scFv (VH2/VL2 in blue) was fused to human Fc with K409D:K392D mutations. (b) The total ion chromatogram (TIC) of deglycosylated bispecific scFv-Fc heterodimer using PNGase F. Both heterodimer and PNGase F peaks were seen in the 55 minute gradient using a Diphenyl. The deconvoluted mass spectrum result of deglycosylated bispecific scFv-Fc heterodimer is shown in the lower panel. The heterodimer with average mass of 107468.74 is seen, and no homodimers were detected in the mass analysis. The calculated heterodimer average mass is 107466.21. (c) The binding of bispecific TARTKxCD3 scFv-Fc heterodimer to U87/TARTK cells and T cells (J45.01) by FACS. OKT3 huLG1 and TARTK huLG1 were used as controls. (d) Specific cytotoxic activity of bispecific TARTKxCD3 scFv-Fc on TARTK expressing cells. U87/TARTK cells (left panel) or U87 cells (right panel) were incubated with PBMC in the presence of
serial dilutions of bispecific TARTKxCD3 scFv-Fc heterodimer (red line), a control bispecific scFv-Fc heterodimer that bind to CD3 and an unrelated antigen (green line), and anti-TARTK huIgG1 (blue line) for 48 hours. Percent of cell lysis was calculated and plotted. (e) Dose-dependent effect of bispecific TARTKxCD3 scFv-Fc heterodimer on the outgrowth of U87-TARTK glioma in NOD/SCID mice. U87/TARTK tumor cells were mixed 1:1 with purified Tcells and innoculated s.c.. The indicated dose of bispecific TARTKxCD3 scFv-Fc heterodimer or DPBS control were administrated via tail vein injections on day 0 and day 4. Tumor growth curve derived from 10 animals were shown.

Figure 4. (a) Schematic drawing of the monovalent IgG (left panel) and SDS PAGE analysis of monovalent IgG preparations using WT Fc (middle panel) and heterodimer Fc with charge pair mutations (right panel). The content of culture supernatant (S) and eluted fractions (EL) after protein A column of each preparation were examined under reduced condition. The bands corresponding to LC-Fc and HC-Fc are pointed by arrows respectively. (b) Anti-mouse TNFR1 14D2 monovalent IgG, either as human Fc (blue) or mouse Fc heterodimer (red), exhibited good potency in blocking TNF mediated cytotoxicity in L929 assays. 14D2 mouse IgG1 was used as a control. IC50 value were estimated from the percentage cell lysis curves and listed. (c) 14D2 monovalent IgGs were free of agonistic activity. The cellular viability of L929 cells was assessed by fluorescence readout in the presence of 14D2 monovalent IgG, either as human Fc (blue) or mouse Fc heterodimer (red), and 14D2 mouse IgG1 and control IgG (anti-Flag) without TNF. (d) 14D2 monovalent IgGs had similar pharmacokinetics profile as full IgG in mouse serum.
Table 1: Complementary Charged Residue Pairs at the CH3 Domain Interface and their Structural Properties

| Residue in Chain A | --- | Residue in Chain B |
|--------------------|-----|--------------------|
| ASP A 356 (30.7%, 0.99Å²) | --- | LYS B 439’ (32.9%, 1.99Å²) |
| GLU A 357 (0.0%, 1.28Å²) | --- | LYS B 370’ (13.4%, 0.94Å²) |
| LYS A 392 (39.2%, 1.21Å²) | --- | ASP B 399’ (7.9%, 0.79Å²) |
| LYS A 409 (0.6%, 0.91Å²) | --- | ASP B 399’ (7.9%, 0.79Å²) |

*aSolvent Accessible Surface Area (ASA, in %), calculated using the available highest resolution Fc crystal structure (1L6X), and Root-Mean-Square-Deviation (RMSD, in Å²) of the side chain atoms, calculated using the identified 46 Fc crystal structures, are shown in parenthesis. ASA < 10% and RMSD > 1.0Å² are highlighted in bold face.
Charge interaction favors heterodimer formation

Charge interaction favors homodimer formation

Charge interaction disfavors homodimer formation

(a) (b) WT (heterodimer = homodimer)

(d) WT (heterodimer = homodimer)

(e) K409D & D399'K (heterodimer >> homodimer)

Figure 1
Figure 2

(a)

(b)

(c)

(d)

|        | WT | D399R | D399K | WT | D399R | D399K | WT | D399R | D399K | D399K | D399K | D399K | D399K |
|--------|----|-------|-------|----|-------|-------|----|-------|-------|-------|-------|-------|-------|
| scFv-Fc| Fc | -     | -     | -  | -     | -     | -  | -     | -     | -     | -     | -     | -     |
| scFv-Fc/Fc | - | -     | -     | WT | K409E | K409D | WT | K409E | K409D | K409D | K409D | K409D | K409D |

|        | scFv-Fc | WT | D399R | D399K | WT | K409E | K409D | WT | K409E | K409D | K409D | K409D | K409D |
|--------|---------|----|-------|-------|----|-------|-------|----|-------|-------|-------|-------|-------|
| scFv-Fc | Fc       | WT | -     | -     | -  | -     | E356K | -  | -     | E356K | -     | -     | E356K |
| scFv-Fc/Fc | Fc/Fc | WT | -     | -     | -  | -     | E356K | -  | -     | E356K | -     | -     | E356K |

|        | scFv-Fc | WT | D399R | D399K | WT | K409E | K409D | WT | K409E | K409D | K409D | K409D | K409D |
|--------|---------|----|-------|-------|----|-------|-------|----|-------|-------|-------|-------|-------|
| scFv-Fc | Fc       | WT | -     | -     | -  | -     | E356K | -  | -     | E356K | -     | -     | E356K |
| scFv-Fc/Fc | Fc/Fc | WT | -     | -     | -  | -     | E356K | -  | -     | E356K | -     | -     | E356K |

Figure 2
Figure 3

(a) CD3 scFv  TARTK scFv

(b) 

(c) Binding to J45.01 cells (huCD3)

(d) U87/TARTK cells

(e) Binding to U87 TARTK+ cells

Heterodimer

Expected Heterodimer mass: 107466.21Da

98% Heterodimer

98% Heterodimer
**Figure 4**

(a) Comparison of Fc domains: CH1, CH2, CH3, VH, VL, CL, S, EL.

(b) Graph showing percent inhibition of cytotoxicity vs. antibody concentration (log M).

(c) Bar graph comparing untreated control cells and treated cells with different antibody concentrations.

(d) Graph showing concentration (ng/ml) vs. time (hours) for different antibody treatments.
Enhancing antibody Fc heterodimer formation through electrostatic steering effects: Applications to bispecific molecules and monovalent IgG
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