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IgG Subclass Variation of a Monoclonal Antibody Binding to Human Fc-Gamma Receptors

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ABSTRACT

The importance of human Fc receptors in immune regulation is well known. Their role is critical not only in the recruitment of cellular effector functions but also in regulating the balance in the periphery between autoimmunity and tolerance. Despite their central importance, there is a dearth of literature on controlled numeric comparisons in affinities of antibody subclasses for gamma receptors. To date, no studies have directly compared humanized antibodies with the same variable region and differing Fc region subclasses which would rule out any differences that may be attributed to variations in the variable region. In this study we characterized the interaction between four humanized monoclonal antibodies; IgG_1, G_2, G_3 and G_4, each possessing an identical variable region and the repertoire of human Fc-gamma (Fcγ) receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB). The studies were performed using both Surface Plasmon Resonance (SPR) and Enzyme-Linked Immunosorbent-Assay (ELISA) formats. The affinities of the antibodies for their antigen molecule, an endogenous human protein, were also analyzed by SPR. While the identity of the Fc-region had no significant effect on the binding to antigen, substantially different affinities for each of the Fcγ receptors, FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB were observed across the various Fc-subclasses.

Keywords: FC-Gamma Receptors, Surface Plasmon Resonance (SPR), Monoclonal Antibodies, IgG Subclass, Affinity, ELISA

1. INTRODUCTION

Monoclonal Antibodies (mAbs) are a rapidly growing class of highly specific therapeutics (Stockwin and Holmes 2003; Piggee 2008; Carter 2006) which, over the last three decades, have become effective treatments for immunological, oncological, transplantation, cardiovascular and infectious diseases (Nissim and Chernajovsky, 2008; Zhang *et al*., 2007). Currently there are more than 20 FDA approved antibody therapeutics on the market, all of which are of the Immunoglobulin G (IgG) class. An IgG is comprised of two light chains each consisting of variable and constant domains and two heavy chains, each consisting of one variable and 3 constant domains. The two heavy chains are linked to each other and to a light chain each by disulfide bonds.

Through advancements in engineering know-how, biopharmaceutically desired characteristics such as affinity, avidity, half-life and effector functions of an antibody can be manipulated (Hudson and Souriau, 2003; Chowdhury and Wu 2005; Stavenhagen *et al*., 2007; Horton *et al*., 2008; Zalevsky *et al*., 2009). For example, a triple mutation (M252Y/S254T/T256E) inserted into the C_H2 domain of a human IgG molecule increased its binding by approximately 10-fold to the human neonatal receptor FcRn with almost a 4-fold increase in serum half-life (Oganesyan *et al*., 2009)

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while other changes in the Fc domain of IgG have yielded a greater than 100-fold improvement in Antibody-Dependent Cellular Cytotoxicity (ADCC) (Stavenhagen *et al*., 2007). Many of the approved therapeutic mAbs are of the IgG_1 subclass, reviewed by Carter (2006). Advantages of IgG_1 include a characteristic longer half-life and the ability to orchestrate immune mediated effector functions (Natsume *et al*., 2009; Strome *et al*., 2007).

IgG Fc receptors play an important role in the control of effector functions of mAbs (Sisto *et al*., 2009) including ADCC (Fanger *et al*., 1989), complement activation, phagocytosis (Anderson *et al*., 1990), release of inflammatory mediators (Anegon *et al*., 1998), antibody production (Fridman, 1993) and immune complex clearance. Three functionally and structurally distinct types of Fcγ-Receptors (FcγR) are expressed on human leukocytes, namely: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). The latter two classes are further divided into FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb. All FcγRs belong to the immunoglobulin superfamily and differ in their antibody affinities. FcγRI has a higher affinity for IgG (K_a = 10^8-$10^9 M^{-1}$), than FcγRII (K_a<$10^7 M^{-1}$) or FcγRIII (K_a <$3\times10^7 M^{-1}$) reviewed by Gessner *et al*., (1998). FcγRI has three immunoglobulin domains in the extracellular portion whereas FcγRII and FcγRIII have two. It is this third domain of FcγRI which confers high affinity and the ability to bind monomeric IgG (Gessner *et al*., 1998; Allen and Seed 1989). In contrast, the lower affinities of FcγRII and FcγRIII for IgG renders these receptors suited to activation through the avidity afforded by the association with multimeric immune complexes (Shields *et al*., 2001). Ligation of FcγRs produces activating signals as with FcγRI and FcγRIII, or inhibitory signals as with FcγRIIb, both of which are integral to a balanced immune response (Nimmerjahn and Ravetch, 2005). FcγRs bind to the lower hinge region of IgG and in the case of IgG_1, a common set of residues appears to be involved in the binding to all FcγRs (Sautes *et al*., 2003; Shields *et al*., 2001).

While the various subclasses of IgGs have distinct selectivity profiles for the Fcγ receptor repertoire (Salfield 2007; Presta *et al*., 2002), most of the supporting studies feature qualitative rankings of FcγRs functional association (Strome *et al*., 2007; Nimmerjahn and Ravetch 2005; Sorge *et al*., 2003). Few studies have shown comprehensive numerical affinities for antibody subclasses binding to the human FcγRs. One study has reported binding of IgG_1 with RIIa, RIIb and RIII only (Maenaka *et al*., 2001). Another study by Bruhns *et al*. (2009) discussed the specificity and affinity of FcγRs and their polymorphic variants to different human IgG subclasses, using purchased chimeric monoclonal and polyclonal antibodies. However, no studies to date have compared human antibodies with the same variable region in combination with the differing Fc subclasses.

Obtaining accurate affinities of each subclass for various FcγRs and understanding the importance of immune complex clearance is important in the design of antibody-based therapeutics. For example, this can allow monoclonal antibodies to be specifically engineered to manipulate clearance. In this study four recombinant antibodies each possessing an identical variable region and differing only in the subclass of Fc-region (G_1, G_2, G_3 and G_4) were produced and shown to be structurally and functionally indistinguishable with respect to the variable region and interaction with the antigen protein. These antibodies were evaluated for binding to each of the FcγRs: FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB using both a monovalent binding SPR format and a multivalent ELISA.

2. MATERIALS AND METHODS

2.1. Antibody and Protein Reagents

Human Fc gamma receptors were purchased from R and D systems; (Cat# FcγRI-1257-FC, FcγRIIA-1330-CD, FcγRIIB-1875-CD, FcγRIIIA-4325-FC and FcγRIIIB-1597-FC/CF). Anti-Fc (goat anti-human-Fc IgG antibody, 1 mg mL^{-1}) was obtained from KPL (Cat#01-10-20). The endogenous human protein antigen was obtained from commercial sources.

2.2. Generation of Purified mAbs

Monoclonal antibodies to the endogenous human protein antigen containing either G_1, G_2, G_3 or G_4 constant regions and the same variable domain were generated using standard molecular biology methods. Plasmids were transfected into CHO K1 cells and cell lines established using single cell cloning (CHO-GS used under license from Lonza Biologics plc.). Antibodies were purified from cell culture supernatants using protein A affinity chromatography. The structural differences between each of the mAb subclasses are well known (Wypych *et al*., 2008) and include the number of disulfide bonds in the hinge region, the location of the heavy and light chain disulfide bonds and the approximately 5% overall primary sequence divergence between the Fc-regions.

2.3. IEF

Purified antibodies (10 µg each) were run on a pH 3-10 Invitrogen IEF gel (Cat#EC6655BOX) and calibrated with an IEF pH 3-10 Invitrogen Serva marker kit (Cat#39212-01). Gels were stained with Coomassie brilliant blue R-250 (Research Organics Cat#1447C).

2.4. Electrospray ToF

The monoclonal antibodies were injected into an Agilent 1100 HPLC and the LC effluent electrosprayed into the Agilent LC/MSD ESI-ToF mass spectrometer operated in positive ion mode. A Vydac C4 reverse phase column (1.0×250 mm, 5 µm particles, 300 Å pore size) was used with a mobile phase A of 94.9% Water, 5% Acetonitrile, 0.1% Trifluoroacetic Acid (TFA) and mobile phase B of 79.9% Acetonitrile, 20% Water, 0.1% TFA. An LC-MS run time of 35 min. was used with a 1 min ballistic desalting gradient from 20-100% B 1 min post injection. MS data were generated with Mass Hunter acquisition software and processed using Mass Hunter Qualitative with BioConfirm deconvolution software to resolve the charge state envelope for each sample and to determine the mass of the intact antibody and any variant structures present. The calibration check spectra were acquired pre-acquisition and post-acquisition of the samples, using ES-ToF Tuning mix.

2.5. Biacore Analysis

Kinetic data were obtained by surface plasmon resonance performed on a Biacore 3000 biosensor (Biacore AB, Uppsala, Sweden). The CM5 sensor chips (research grade), amine coupling reagents (NHS, EDC, ethanolamine pH 8.5, HBS-EP buffer, 10 mM sodium acetate buffer (pH 5.0) and P20 were obtained from Biacore AB. The binding kinetics of mAbs to the antigen was determined by a capture approach using single cycle kinetics (Karlsson *et al*., 2006). In this approach, the CM5 sensor chip was normalized and primed using fresh degassed/filtered HBS-EP buffer prior to anti-Fc mAb immobilization at 25°C on two flow cells of the chip at a concentration of 0.1 mg mL^{-1} in 10 mM sodium acetate pH 5.0 for 8 min. at 10 µL min^{-1} via amide coupling chemistry. The mAbs were diluted between 0.8 and 1.4 µg mL^{-1} and, in separate experiments, injected as follows: IgG_1- 20 µL, IgG_2-30 µL, IgG_3-18 µL, IgG_4-20 µL at 20 µL min^{-1}. Concentrations of 0.375, 0.75, 1.5, 3 and 6 nM antigen in HBS-EP were injected over the Anti-FC/mAb surface in single cycle kinetics mode. Experiments were run at 25°C sensor surface temperature. Data were analyzed using a titration kinetics 1:1 Model in BIAsimulation software (Biacore AB, Uppsala, Sweden). The binding affinities of the mAbs to Fcγ receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB) were determined by directly immobilizing the mAbs to the sensor surface. The mAbs diluted in 10 mM sodium acetate pH 5 were immobilized to one flow cell; the other flow cell was immobilized as a blank reference. Immobilization levels were optimized to show sufficient binding levels of receptors. Various concentrations of receptors were analyzed in HBS-EP. Experiments were run either by single cycle kinetics mode or steady state equilibrium depending on initial affinities in experimental scouting. Data were analyzed using a Steady State Affinity Model in BIAsimulation software (Biacore AB, Uppsala, Sweden). **Table 1** outlines concentrations of antibody used in immobilization (including resonance units immobilized), receptor concentration and experimental mode.

Table 1. Methods summary

FcγR	mAb Concn. µg/mL	RU's mAb Immobilized	FcγR Concentrations (nM)	Expt Mode
Anti-) Fc (G1	0.8	318	0.19, 0.38,0.75,1.5,3.0	SCK[a]
Anti-) Fc (G2	62	1570	31,63,125,250,500,1000,2000	SSE[b]
Anti- Fc (G3)	0.1	318	0.19, 0.38,0.75,1.5,3.0	SCK[a]
Anti- Fc (G4)	0.7	338	0.19, 0.38,0.75,1.5,3.0	SCK[a]
FcγRIIA				
Anti- Fc (G1)	1.6	542	24,49,98,195,391,781,1563,3125	SSE[b]
Anti- Fc (G2)	62	1569	24,49,98,195,391,781,1563,3125	SSE[b]
Anti- Fc (G3)	2.1	547	24,49,98,195,391,781,1563,3125	SSE[b]
Anti- Fc (G4)	1.4	546	24,49,98,195,391,781,1563,3125	SSE[b]
FcγRIIB				
Anti- Fc (G1)	81.1	2584	26,52,104,208,417,833,1667,3333,6666	SSE[b]
Anti- Fc (G2)	62	1569	26,52,104,208,417,833,1667,3333,6666	SSE[b]
Anti- Fc (G3)	2.1	542	26,52,104,208,417,833,1667,3333	SSE[b]
Anti- Fc (G4)	1.4	546	26,52,104,208,417,833,1667,3333	SSE[b]
FcγRIIIA				
Anti- Fc (G1)	1.6	524	9,17,34,69,139,278,556,1111,2222,4444	SSE[b]
Anti- Fc (G2)	62	1569	9,17,34,69,139,278,556,1111,2222	SSE[b]
Anti- Fc (G3)	2.1	547	9,17,34,69,139,278,556,1111,2222	SSE[b]
Anti- Fc (G4)	1.4	546	9,17,34,69,139,278 ,556,1111,2222,4444	SSE[b]
FcγRIIIB				
Anti- Fc (G1)	1.6	542	16,31,63,125,250,500,100,2000	SSE[b]
Anti- Fc (G2)	62	18171	16,31,63,125,250,500,100,2000	SSE[b]
Anti- Fc (G3)	2.1	546	16,31,63,125,250,500,100,2000	SSE[b]
Anti- Fc (G4)	137	16098	16,31,63,125,250,500,100,2000	SSE[b]

Single Cycle Kinetics[a], Steady State Equilibrium[b]

2.6. Binding of Antibodies to FcγR by ELISA

Ni-NTA pre-coated plates (Qiagen, Cat#35061) were incubated with 50 µL well^{-1} of His-tagged human FcγR I, IIb/c or III (R and D systems Cat#s1257-FC, 1875-CD, 4325-FC) at a receptor concentration of 5 µg mL^{-1} in PBS, overnight at 4°C. Following overnight incubation with receptor, the plates were washed 3 times with PBS buffer containing 0.02% Tween-20 using a multiwash advantage plate washer. After washing, 50 µL of complexed antibodies were diluted in PBS buffer containing 0.05% Tween-20 and incubated in the plate for 60 min. at room temperature. Complexed antibody was prepared by prior incubation of antibodies overnight with a biotinylated F(ab')$_2$ fraction of goat-anti-human F(ab')$_2$ (Jackson Immunolabs Cat# 109-066-006), at a 2:1 antibody: F(ab')$_2$ molar ratio in PBS. Following incubation of the plate wells with complexed antibody, the plates were washed as described above and followed by the addition of 50 µL well^{-1} of the secondary antibody streptavidin-HRP to detect biotinylated complexed antibodies (Zymed Cat#43-4323, diluted 1/2000 in PBS buffer containing 0.05% Tween-20). Incubation with secondary antibody was for 60 min. at room temperature. Following this incubation, wells were washed and color development initiated with 100 µL well^{-1} of *o*-phenylenediamine dihydrochloride (OPD, Sigma Cat# P 9187). Reactions were stopped with 25 µL well^{-1} 12.5% H_2SO_4 and the absorbance read at 490 nm.

3. RESULTS

3.1. Physical and Structural Characterization of Recombinantly Expressed Monoclonal Antibody Subclass Variants

IEF analysis of the antibodies used in this study is shown in **Fig. 1**, from which the following pI values were obtained: IgG_1 = 7.9; IgG_2 = 7.1; IgG_3 = 8.0; IgG_4 = 6.7. The appearance of multiple bands most likely reflects heterogeneity in post translational modifications. The electrospray ToF evaluation revealed a considerable difference in the molecular weight of the various subclasses in accordance with differences in the amino acid sequence and number of disulfide bonds (**Fig. 2**). The experimentally determined molecular weights were: IgG_1 = 148,475(±17 ppm); IgG_2 = 147,982(±24 ppm); IgG_3 = 158,668(±21 ppm); IgG_4 = 148,025(±21 ppm), with error calculated from a theoretically determined mass. The higher molecular weight of IgG_3 compared to the other IgG subclasses is attributed to the elongated hinge region.

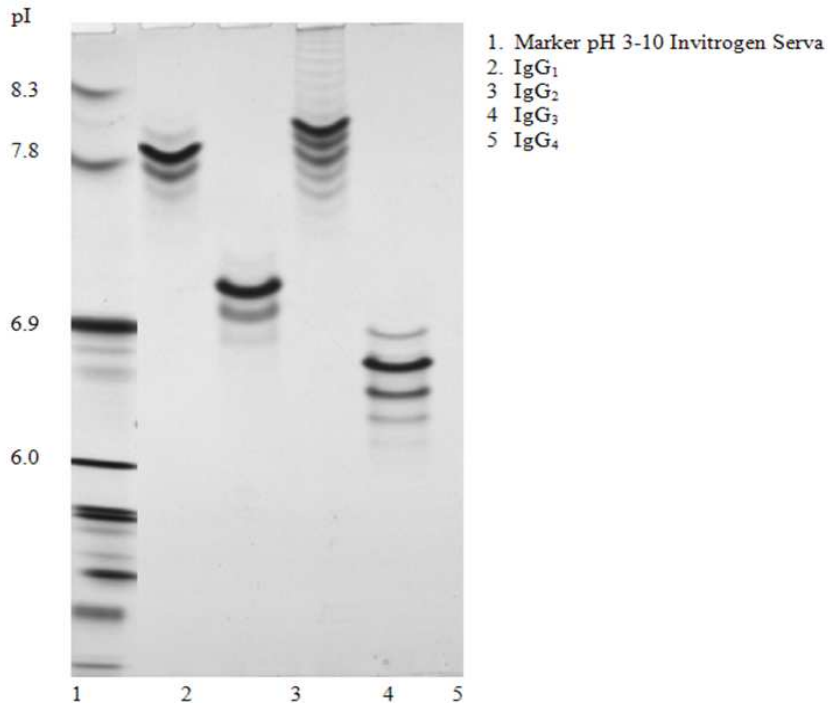
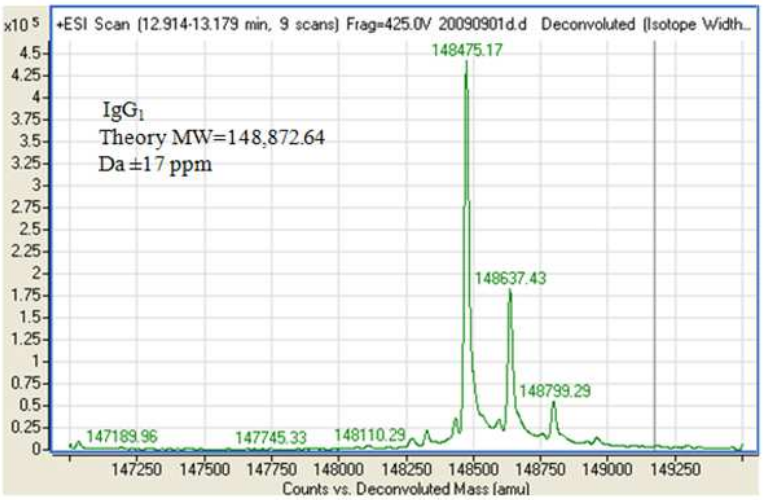
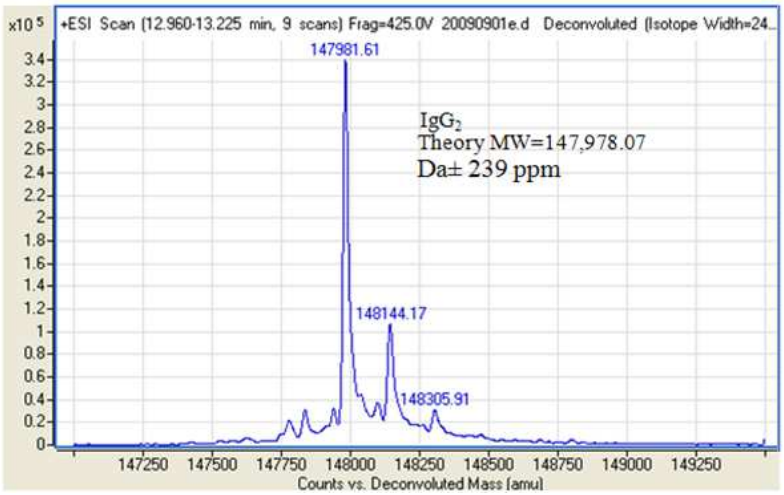
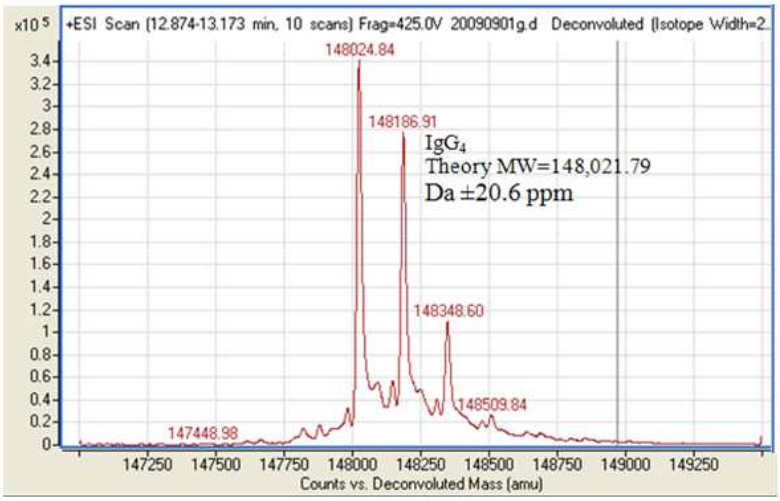


Fig. 1. The heterogeneity of mAb subclasses is shown by vertical IEF. The following pI values were


x10 5
+ESI Scan (12.914-13.179 min, 9 scans) Frag=425.0V 20090901d.d Deconvoluted (Isotope Width...
148475.17
IgG1
Theory MW=148,872.64
Da ±17 ppm
148637.43
148799.29
147189.96
147745.33
148110.29
Counts vs. Deconvoluted Mass (amu)


x10 5
+ESI Scan (12.960-13.225 min, 9 scans) Frag=425.0V 20090901e.d Deconvoluted (Isotope Width=24...
147981.61
IgG2
Theory MW=147,978.07
Da± 239 ppm
148144.17
148305.91
Counts vs. Deconvoluted Mass (amu)


x10 5
+ESI Scan (12.874-13.173 min, 10 scans) Frag=425.0V 20090901g.d Deconvoluted (Isotope Width=2...
148024.84
148186.91
IgG4
Theory MW=148,021.79
Da ±20.6 ppm
148348.60
148509.84
147448.98
Counts vs. Deconvoluted Mass (amu)

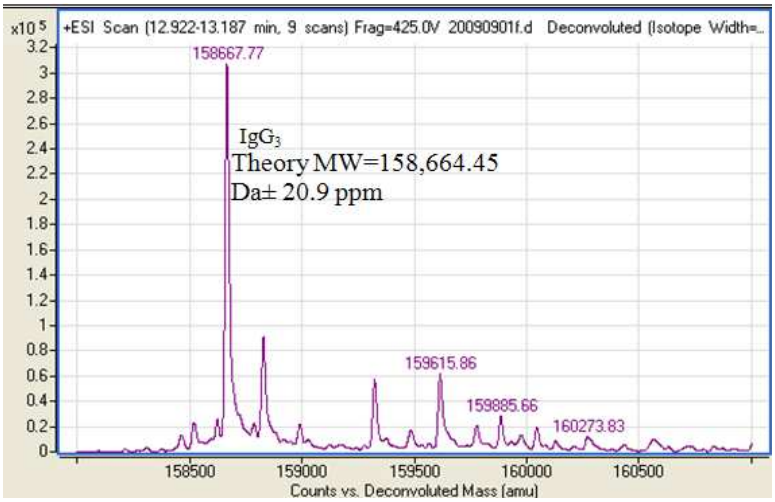
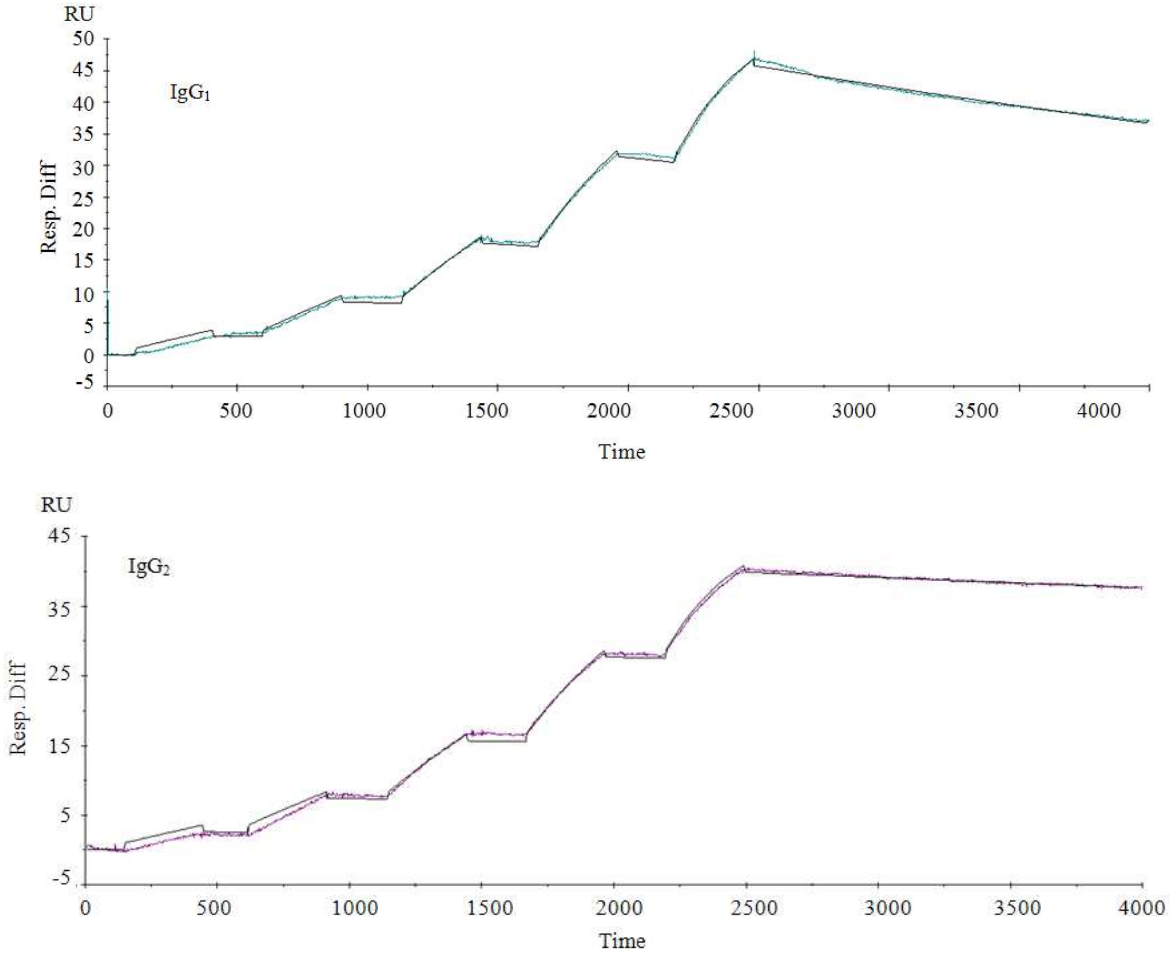


Fig. 2. Intact molecular weights of each of the IgG subclasses evaluated by Electrospray ToF. The experimentally determined molecular weights were: IgG_1 = 148,475 (±17 ppm); IgG_2 = 147,982(±24 ppm); IgG_3 = 158,668(±21 ppm); IgG_4 = 148,025 (±21 ppm)



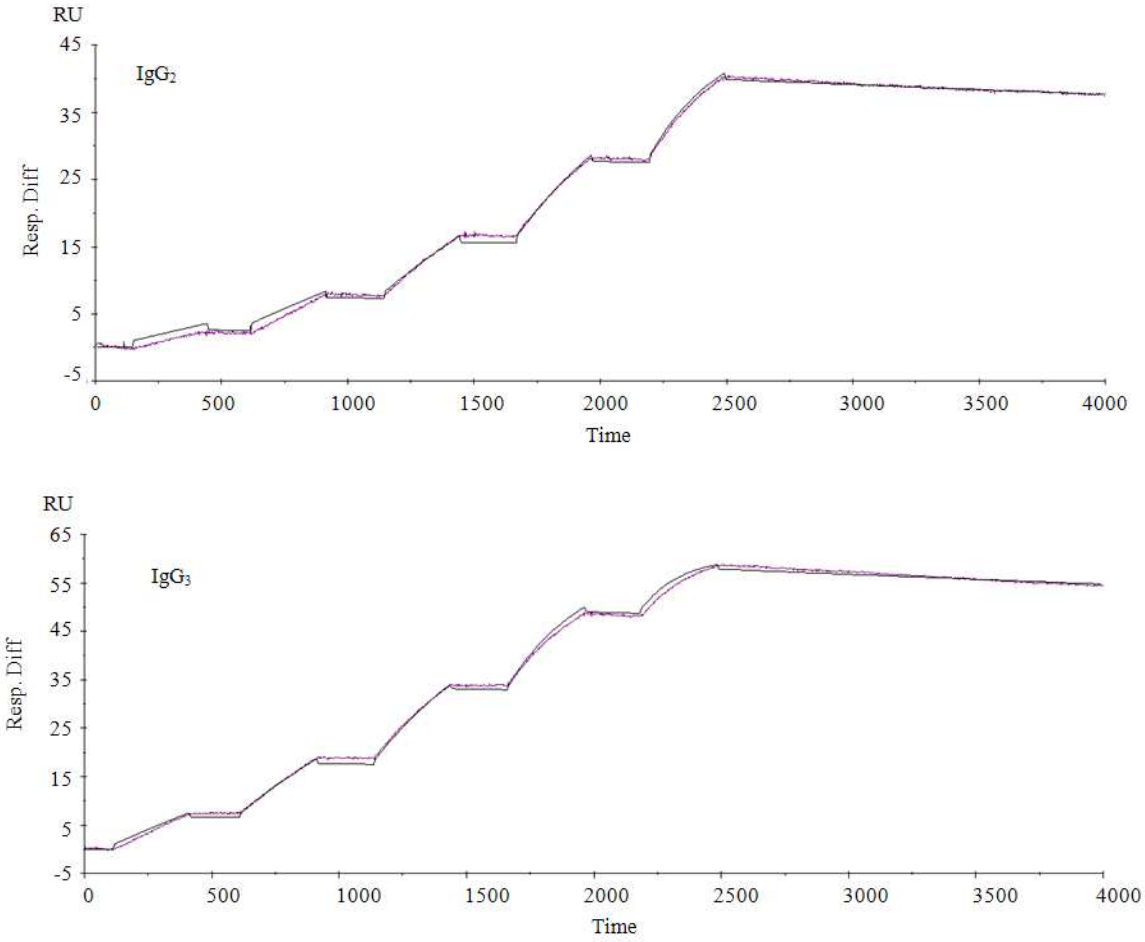
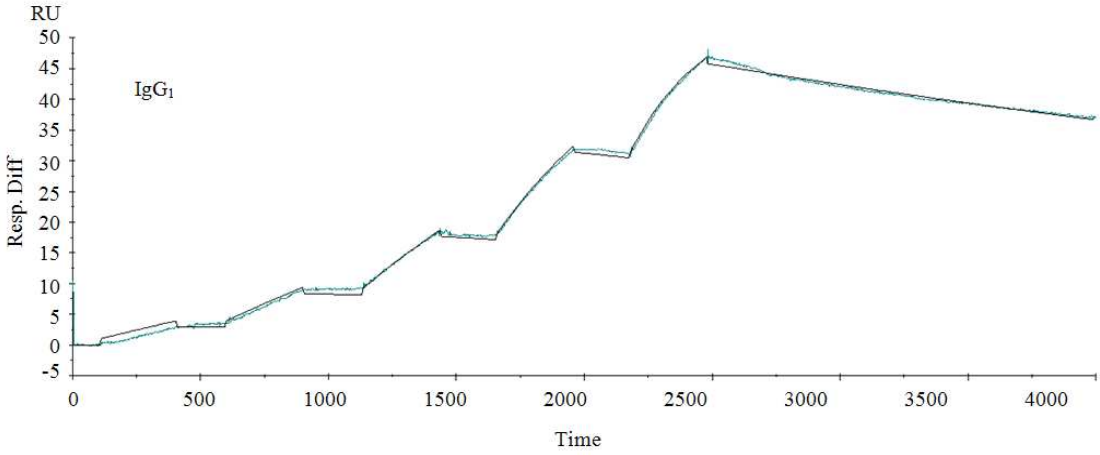


Fig. 3. Sensorgrams of mAb subclasses ($G_{1,}$ $G_{2,}$ $G_{3\ and}$ $G_{4)}$ binding to antigen using a single cycle kinetics technique by Biacore. Each step in the sensorgram represents increasing concentration of antigen. The final concentration/step shows both association and dissociation of antibody binding to antigen



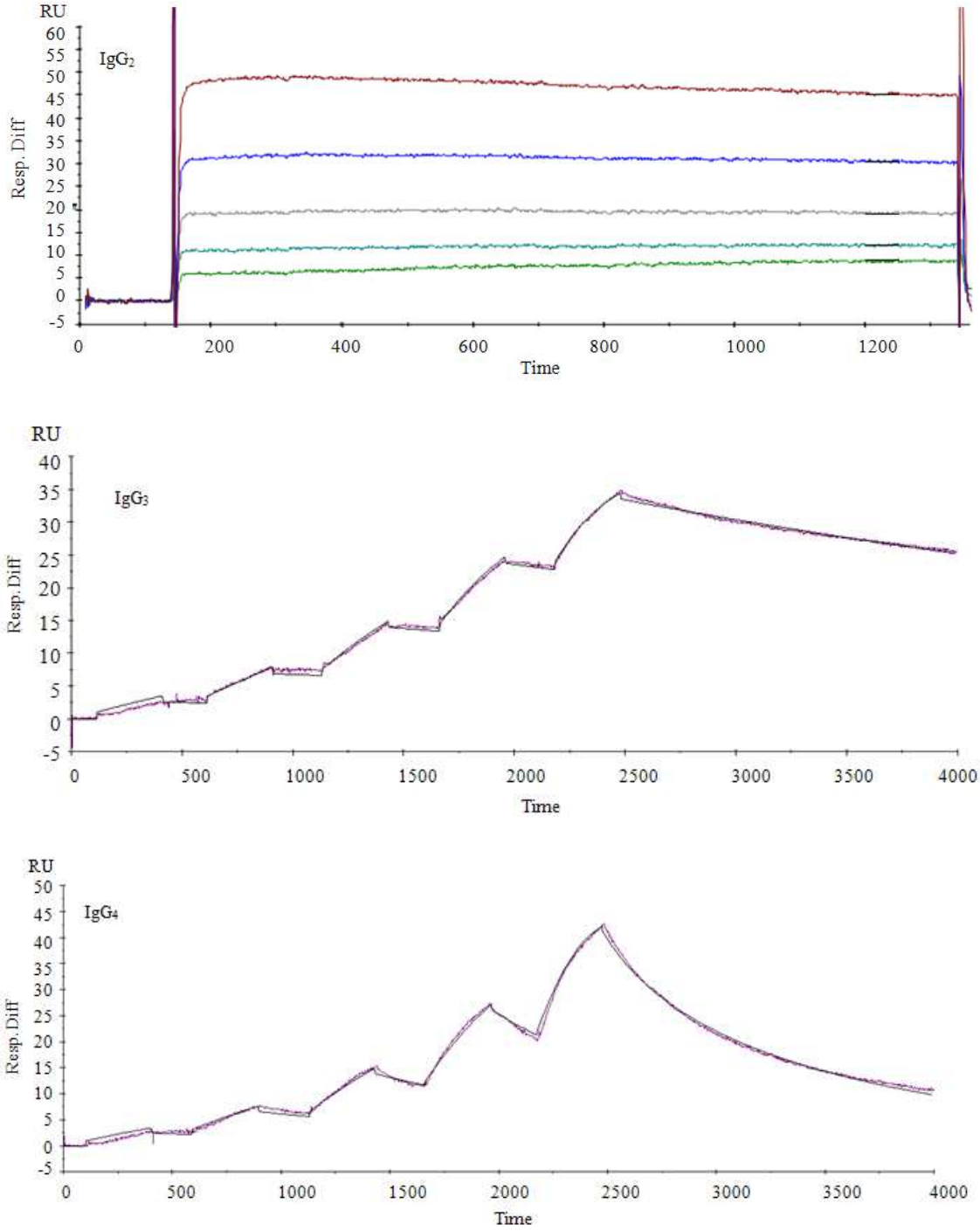


Fig. 4. Sensorgrams of mAb subclasses (G_1, G_2, G_3 and G_4) binding to FcγRI receptor by Biacore. mAb subclasses (G_1, G_3 and G_4) binding to FcγRI evaluated by single cycle kinetics. Each step represents increasing concentration of FcγRI. The final step/concentration shows both association and dissociation of the antibody binding to FcγRI. IgG_2 was evaluated by steady state eqilibrium. Each step represents increasing concentration of FcγRI Receptor

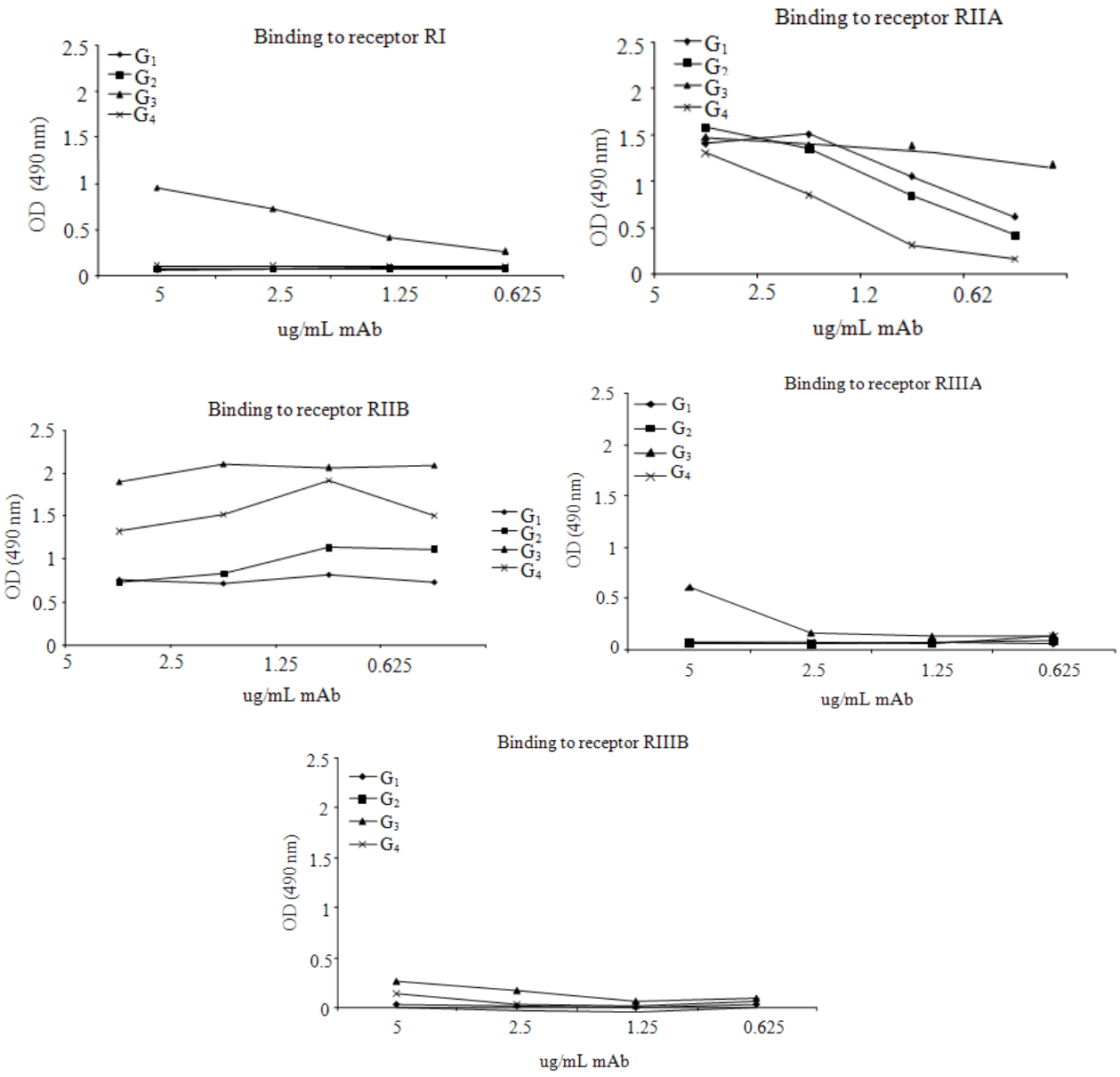


Fig. 5. Binding of cross linked humanized antibody subclasses (G_1,G_2,G_3 and G_4) to various Fcγ Receptors as immune complexes with F(ab')$_2$-anti-F(ab')$_2$, using an ELISA format. All IgG subtypes bind well to FcγRIIA and FcγRIIB, the inhibitory FcγR. IgG_3 binds to all FcγRs

Table 2. Kinetics of mAbs (G_1, G_2, G_3 and G_4) binding to antigen by Biacore

mAb Subclass	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
IgG_1	6.91E+05	2.62E-05	3.80E-11
IgG_2	6.82E+05	4.01E-05	5.88E-11
IgG_3	1.05E+06	3.63E-05	3.45E-11
IgG_4	6.94E+05	2.81E-05	4.04E-11

Table 3. Binding affinities of mAbs with different Fc regions to human Fc-gamma receptors by Biacore

mAb subclass	Receptor FcγRI K_D(M)	Receptor FcγRIIA K_D(M)	Receptor FcγRIIB K_D(M)	Receptor FcγRIIIA K_D(M)	Receptor FcγRIIIB K_D(M)
IgG_1	1.23E-10	8.00E-07	3.10E-06	8.50E-07	1.90E-06
IgG_2	1.40E-06	3.78E-07	6.80E-06	2.20E-06	ND[a]
IgG_3	7.90E-11	8.97E-08	1.30E-06	3.90E-07	1.44E-06
IgG_4	6.90E-10	6.00E-07	1.70E-06	3.46E-06	4.60E-06

Table 4. Affinity ranking for the binding of mAbs with varying Fc regions to Fc-gamma receptors as determined by Biacore and an ELISA format

Fc-receptor type	Rank order by SPR	Rank order by ELISA
FcγRI	IgG_2<<IgG_4<IgG_1<IgG_3	IgG_3
Fcγ RIIA	IgG_1~IgG_4<<IgG_2<IgG_3	IgG_4<IgG_2 <IgG_1<IgG_3
Fcγ RIIB	IgG_1~IgG_2~IgG_3~IgG_4	IgG_1~ IgG_2~IgG_3~IgG_4
Fcγ RIIIA	IgG_2~IgG_4<IgG_1<IgG_3	IgG_3 (bound poorly)
Fcγ RIIIB	IgG_1~IgG_2~IgG_3~IgG_4	IgG_4< IgG_3 (both bound poorly)

3.2. Binding of the Monoclonal Antibody Subclass Variants to the Antigen Protein

The sensorgrams from single cycle kinetics are shown in **Fig. 3**. All four subclass variant antibodies bound with high affinity to the endogenous human protein antigen. Negligible difference was observed in K_D values among the variants (**Table 2**). The observed K_D values were: IgG_1 = 38 pM, IgG_2 = 59 pM, IgG_3 = 35 pM, IgG_4 = 40 pM (**Table 2**). The association (k_a) and dissociation (k_d) rate constants were also similar. These data clearly show that changes in the Fc type do not result in conformational changes in the variable region that affect antigen protein binding.

3.2. Affinity of Binding of the Monoclonal Antibody Subclass Variants to FcγR:

The binding affinities of the four subclass variant antibodies to each of FcγRI, FcγRIIA (R131), FcγRIIB, FcγRIIIA (V158) and FcγRIIIB as determined by SPR were significantly different (**Table 3**). As an example the sensorgrams of FcγRI binding to each mAb are shown in **Fig. 4** and the affinity rankings derived from all of the single cycle kinetics and steady state equilibrium experiments for all mAbs binding to each of the gamma receptors are summarized in **Table 4**. With the exception of IgG_2, the subclass variants had the strongest affinity for FcγRI with the following K_D values: IgG_1 = 123 pM, IgG_3 = 79 pM, IgG_4 = 690 pM. IgG_2 had the strongest affinity for Receptor FcγRIIA (378nM). IgG_3 had comparatively high affinity for FcγRIIA (K_D =90 nM) and FcγRIIIA (K_D = 390 nM). The binding affinities for all other receptor-antibody binding combinations were in the much weaker micromolar range.

3.3. Avidity driven Binding of the Monoclonal Antibody Subclass Variants to FcγR

Multivalent immune complexes were generated by cross linking each mAb with a $F(ab')_2$ fraction of goat-anti-human $F(ab')_2$. The avidity of the complexed mAbs for binding to each surface immobilized FcR was determined by ELISA (**Fig. 5**). The complexed mAbs were all able to bind to FcγRIIB, the inhibitory receptor, whereas FcγRI only bound IgG_3. FcγRIIA bound all subclasses, with G_3> G_1> G_2>G_4. FcγRIIIB showed minimal binding to IgG_3, (**Table 4**).

4. DISCUSSION

These studies are the first to evaluate FcγR binding to all IgG subclasses using functional humanized mAbs with identical variable regions. Several other studies have evaluated the binding of particular subclasses to some of these receptors including a study by Maenaka *et al*. (2001) where the binding of Fcγ receptors RIIA, RIIB and RIII to IgG_1 was evaluated. Bruhns *et al*., (2009) undertook a comprehensive assessment of the relationship between mAb subclass and binding to FcRs that also incorporated the consideration of receptor polymorphism, but the study used mouse/human chimeric monoclonal and polyclonal antibodies.

Monovalent binding of Fc receptors and the mAbs, as measured by SPR, indicated affinities for FcγRI in the high pM range with G_3 having the highest affinity, followed by G_1. These affinities were stronger than those observed by Canfield and Morrison (1991) and Gessner *et al*. (1998) although the same rank order was observed in each case. Interaction of monovalent antibodies of each subclass with the low affinity FcγRII and FcγRIII receptors, which normally rely on multivalent complexing, were measurable by SPR in our study with G_3 having the strongest affinity for FcγRIIA and FcγRIIIA (K_D of 89 and 390nM, respectively). This was consistent with Bruhns *et al*., (2009) concerning low affinity FcγRIIIA bound by monomeric G_3. Each of the four subclasses of mAb bound to FcγRI, FcγRIIA, FcγRIIB and FcγRIIIA as determined by SPR. FcγRIIIB, which did not appear to bind to IgG_2 in either monovalent format or multivalent format, was the only exception.

Our study also showed IgG_1 bound human FcγR with affinities (K_D) ranging from pM in the case of

FcγRI (123pM) and FcγRIIA (800nM) to μM as seen with FcγRIIB, FcγRIIIA and FcγRIIIB (all close to 1 μM). The IgG_2 monoclonal antibody also bound FcγR with a narrower range than that seen for IgG_1. Most of the affinities were in the single digit micromolar range, with the exception of FcγRIIA which had an affinity of 0.38μM and FcγRIIIB, which was not determined. IgG_3 was able to bind all FcγR's, with a very broad range of affinities. The strongest affinity was for FcγRI, with a K_D of 79pM. As with IgG_1, IgG_3 had nanomolar affinity for FcγRIIA (90nM). Low affinity receptors FcγRIIB, FcγRIIIA and FcγRIIIB had K_D values in the low micromolar range. IgG_4 exhibited a similar pattern of affinities for all FcγR, with K_D values of 690pM for FcγRI, 600nM for FcγRIIA and values in the low micromolar range for low affinity receptors FcγRIIB, FcγRIIIA and FcγRIIIB.

Overall, SPR assessment of monovalent interactions between humanized IgG and FcγR support published studies by Bruhns *et al*. (2009) in which FcγRI has strong affinity for IgG_1, G_3 and G_4 subtypes, with K_D values in the picomolar range. FcγRIIA has moderate affinity for all subtypes, including IgG_2, with K_D values in the nanomolar range. The remaining FcγRs which were evaluated (FcγRIIB, FcγRIIIA and FcγRIIIB) had affinities in the micromolar range.

FcγRIIB, FcγRIIIA and FcγRIIIB, as well as FcγRIIA are considered low affinity receptors and exert their regulatory functions in a multivalent format, via immune complexing. Affinity rankings of the humanized monoclonal antibodies, in immune complexes with $F(ab)'_2$-anti-$F(ab)'_2$, are compared with monomeric SPR derived affinities in **Table 4**.

In this avidity driven format, IgG_1 and IgG_2 bound to FcγRIIA and FcγRIIB, the inhibitory FcγR. IgG_3 showed association with all FcγRs and was the only subtype which associated with FcγRI and FcγRIIIA. Rank order of IgG_3 and FcγRs show that the strongest affinity is for FcγRIIA followed by FcγRIIB>FcγRI> FcγRIIIA>FcγRIIIB. IgG_4 had no affinity for FcγRI or FcγRIIIA and only marginal association with FcγRIIIB. It did associate strongly with FcγRIIA and FcγRIIB, the inhibitory receptors.

Low affinity, inhibitory receptors, FcγRIIB and FcγRIIA, bound all mAb subclasses, with IgG_3 having a greater binding than IgG_1 in both monomeric and multimeric formats. This is in agreement with Bruhns *et al*. (2009) who also examined interactions both in monomeric and multimeric conditions. The low affinity receptor FcγRIIIA had discernible binding to all IgG subtypes, with K_D values in the micromolar range as determined by SPR. Using similar SPR studies; Bruhns *et al*. (2009) reported affinities for FcγRIIIA with only IgG_1 and IgG_2. In our study using SPR, FcγRIIIB was found to have a weak affinity for IgG_1, IgG_3 and IgG_4. No affinity was seen for IgG_1 or IgG_2 with FcγRIIIB using multimeric ELISA.

5. CONCLUSION

This study evaluated the interaction of four subclass variant antibodies both to the antigen protein and to the repertoire of human FcγRs. Comparable affinities with K_D values ranging between 35 and 59 pM were observed for the binding of all four antibodies to the antigen, showing that the differing Fc regions did not impart conformational changes to the variable region associated with altered antigen protein binding. In contrast, the subclass variants exhibited significantly different affinities for each of the Fcγ receptors FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB. Since the subclass variants each had the exact same VH, VL and CL regions the differences seen were attributable solely to the Fc regions known to be involved in FcγR binding.

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