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Human IgG subclass cross-species reactivity to mouse and cynomolgus monkey Fcγ receptors

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ABSTRACT

In therapeutic antibody discovery and early development, mice and cynomolgus monkey are used as animal models to assess toxicity, efficacy and other properties of candidate molecules. As more candidate antibodies are based on human immunoglobulin (IgG) subclasses, many strategies are pursued to simulate the human system in the test animal. However, translation rate from a successful preclinical trial to an approved drug is extremely low. This may partly be due to differences in interaction of human IgG based candidate molecules to endogenous Fcγ receptors of model animals in comparison to those of human Fcγ receptors. In this study, we compare binding characteristics of human IgG subclasses commonly used in drug development (IgG1, IgG2, IgG4) and their respective Fc silent versions (IgG1σ, IgG2σ, IgG4 PAA) to human, mouse, and cynomolgus monkey Fcγ receptors. To control interactions between Fab and Fc domains, the test IgGs all have the same variable region sequences. We found distinct variations of interaction of human IgG subclasses to model animal Fcγ receptors in comparison to their human counterparts. Particularly, cynomolgus monkey Fcγ receptors showed consistently tighter binding to human IgGs than human Fcγ receptors. Moreover, the presumably Fc silent human IgG4 PAA framework bound to cynomolgus monkey FcγRI with nanomolar affinity while only very weak binding was observed for the human FcγRI. Our results highlighted the need for a thorough *in vitro* affinity characterization of candidate IgGs against model animal Fcγ receptors and careful design of preclinical studies.

1. Introduction

Fcγ receptors are membrane anchored proteins expressed in many immune effector cells and mediate antibody functions [1–3]. They interact with the Fc domain of immunoglobulins (IgGs) via their extracellular domains (ECDs) [4,5] and mediate critical cellular functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) [3]. Most Fcγ receptors have activating functions while one important class (FcγRIIb) is of the inhibitory type. The balance between the activating and inhibitory signals is thought to play a determining role in effector responses [6]. Structurally, the Fcγ receptors share a similar architecture with 2 or 3 extracellular immunoglobulin (Ig)-like domains and an intracellular domain that carries either the activating or the inhibitory motif. Although Fcγ receptors can have high or low affinity to IgGs, they all bind to a common region of the IgG molecule – the upper CH_2 domain/lower hinge region, albeit with intricate variations [4,5,7,8]. These interactions form the basis of antibody engineering in IgG based therapeutic antibody development [4,9,10]. For instance, IgG frameworks with highly attenuated binding to Fcγ receptors are actively being investigated for cancer therapies [11].

In the early stages of antibody based drug development, animal models, the choice of which depend on many factors [12], are used in preclinical studies to evaluate a variety of candidate molecule properties. For instance, mouse models are used to evaluate half-life [13] while Non-human primates (NHPs) are used to evaluate toxicity and pharmacokinetic properties. However, antibodies in development are increasingly based on human IgG frameworks, usually IgG1, IgG2 or IgG4. This means during preclinical trials a human antibody interacts with cellular machinery of model animal, including their Fcγ receptors, which might lead to cross-reactivity and/or misleading readout. This necessitates that either the model animal is chosen to have as close genetic makeup to humans or be transgenically modified.

Fcγ receptors of mice and several NHPs have been investigated [1,14–19], though not well understood as their human counterparts. Mouse Fcγ receptors show significant sequence (Supplementary Fig. 1) and functional variation from those of humans. So, a candidate therapeutic antibody based on a human IgG framework would show significant functional divergence in wild type mice. To mitigate this, mice models that mimic the human system as close as possible have been

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developed [20]. In contrast, Fcγ receptors of NHPs share very high sequence similarity to those of humans [21] (Supplementary Fig. 1) and show functional similarities as well but important variations have been observed [14,17,18]. A critical concern is that because NHP model animals are not genetically modified for ethical reasons, a candidate antibody molecule based on a human IgG subclass interacts with endogenous NHP Fcγ receptors, and as a result the functional readout of a preclinical study with a human IgG based therapeutic candidate molecule may have subtle variations to that of the human system. A good example of this is the case of TGN1412, which was deemed safe in animal studies but resulted in life-threatening conditions in human clinical trials [22,23]. Although there might be many reasons for this discrepancy, subtle differences in the interaction of the human IgGs to endogenous model animal Fcγ receptors were thought to be one of the factors. So, a thorough characterization of the interaction of human IgG subclasses, particularly those commonly used in drug development efforts, to model animal Fcγ receptors is necessary to gauge functional readout and carefully design animal experiments.

Bruhns et al. characterized in detail the specificity and affinity of human IgG subclasses to human Fcγ receptors and their polymorphisms [24]. Ironically, although there has been extensive use of mice and NHPs for antibody drug development, affinity studies of human IgG subclasses with endogenous Fcγ receptors of model animals have only been attempted recently [14,15,18,24–27]. These studies typically compare affinities of Fcγ receptors of a model animal to human IgGs to that of endogenous IgGs. Although binding data is useful in a specific context, there are significant variations from study to study. This is not unexpected since binding kinetics may be affected by many factors, which makes comparing values from study to study not straight forward. A key discrepancy is that the variable domain of IgGs from the reported studies are usually different and the variable domain of IgGs can affect binding to Fcγ receptors [5,11]. This study addresses this discrepancy by employing identical variable domain in all the test IgGs and by maintaining identical experimental conditions. Binding kinetics of Fcγ receptors from two of the widely used model animals in IgG based drug development – *Mus musculus*(mouse) and *Macaca fascicularis* (Crab-eating macaque) (herein referred as cynomolgus monkey or cyno) are systematically compared against corresponding representative polymorphisms of human Fcγ receptors.

IgG subclass variants with muted Fc effector function have been sought for some therapeutic antibody platforms such as effector cell redirection programs. Some of these Fc silent human IgG subclasses include IgG2σ (V234A/G237A/P238S/H268A/V309L/A330S/P331S, using the EU numbering system) [28] and IgG4 PAA (S228P/F234A/L235A) [29,30]. A silent human IgG1, termed IgG1σ, was also recently developed with mutations L234A/L235A/G237A/P238S/H268A/A330S/P331S [31]. The premise is that if the IgG does not appreciably bind to Fcγ receptors, it would not elicit receptor mediated cellular functions. When these Fc silent subclasses are tested in model animals, it is assumed that their binding to Fcγ receptors is attenuated similarly to the human Fcγ receptors. However, the observed subtle variations with wild type human IgG molecules would likely manifest with the Fc silent frameworks. Therefore, a thorough characterization of affinity of model animal Fcγ receptors to silent human IgG frameworks is critical to foresee any unintended reactions, to adjust expectations, or to design appropriate preclinical experiments. In this study, binding of mouse and cynomolgus monkey Fcγ receptors to human Fc silent subclasses IgG1σ, IgG2σ and IgG4 PAA are compared side-by-side to that of human Fcγ receptors.

2. Materials and methods

2.1. Protein expression and purification

2.1.1. Antibodies

All test antibodies used in this study (Table 1) were purified in-house. Heavy chain constructs of Abs were designed with α-RSV Fab domain protein sequence and one of human IgG subclass sequence (IgG1, IgG2 IgG4 S228P, IgG1σ, IgG2σ and IgG4 PAA) and assembled in Vector NTI with suitable leader sequences for expression in Expi293 cells. Light chain constructs were also constructed similarly with human kappa sequence. Expi293 cells were transiently co-transfected with heavy chain:light chain DNA using ExpiFectamine 293 transfection kits (Life Technologies) and protocol. After 4–5 days incubation, cultures were harvested by centrifugation and filtered. Supernatants were then loaded onto MabSelect SuRe columns (GE), eluted with citrate buffer pH 3.5 and buffer exchanged to 1X DPBS (pH 7.2) with HiPrep 26/10 desalting column (GE). Peak fractions were combined, filtered, checked by SDS-PAGE and size exclusion chromatography and stored in 4 °C until use. Isotype control antibodies were either purified in-house (murine IgG2a from hybridoma suspension by protein A binding) or purchased from commercial sources (cyno IgG control – MyBioSource).

Table 1
Antibodies (Abs) and Fcγ receptors used in the study.

Test Antibodies (Abs)		Fcγ Receptors (FcγRs)		
Wild type	Silent	Human (selected polymorphisms)	Mouse	Cynomolgus monkey
α-RSV/hu IgG1	α-RSV/hu IgG1σ	I (CD64)	I	I
α-RSV/hu IgG2	α-RSV/hu IgG2σ [28]	IIa (CD32a R131)	IIb	IIa
α-RSV/hu IgG4 S228P [29]	α-RSV/hu IgG4 PAA [29,30]	IIb/c (CD32b/c)	III	IIb
Control Abs		IIIa (CD16a V158)	IV	III
muIgG2a isotype control				
cyIgG isotype control				

Database references are given in Supplementary Table 1. Hu- human, mu-murine (mouse), cy- cynomolgus monkey, WT- wild type.

2.1.2. Fcγ receptors

Fcγ receptors used in this study (Table 1) were purified as described below. Commercial FcγR reagents (R&D systems) were also used to validate the purified proteins. Because the intention was to compare IgG binding to model animal receptors to those of humans, a representative set of human FcγR polymorphisms were selected. Mice and cynomolgus monkey each have four well characterized FcγRs but no polymorphisms were identified to date. As this is an entirely *in vitro* study, soluble receptors of only the extracellular domains (ECDs) were produced.

DNA sequences of ECDs of human, mouse, and cynomolgus monkey Fcγ receptors (UniProt or Pubmed IDs shown in Supplementary Table 1) along with native signal peptides and multi-histidine C-terminal tags were synthesized (Genwiz) and cloned into mammalian expression vectors. Expi293 cells were transiently transfected with the plasmids using ExpiFectamine 293 transfection kits (Life Technologies) and protocol. Cultures were harvested after 4–5 days incubation by centrifugation and filtered. Supernatants were then loaded onto HisTrap HP columns (GE), washed with 20 mM imidazole buffer (pH 7.5) and eluted with 300 mM imidazole buffer (pH 7.5). Fractions were combined, concentrated with Amicon Ultra 3 K MWCO centrifugal filtration devices (Millipore) and purified (buffer exchanged) over a Sepax SRT-10C SEC 300 column in 1X DPBS pH 7.2 buffer. Fractions were combined, checked by SDS-PAGE, flash frozen in liquid nitrogen and stored in a −80 °C freezer until use.

2.2. Binding assays

Three assay platforms were explored for this study: Competition AlphaScreen (Perkin Elmer), BioLayer Interferometry (ForteBio, Pall Life Sciences) and Surface Plasmon Resonance (ProteOn, BioRad). The latter two label-free methods were used to obtain affinity data

presented here. AlphaScreen data is not included for reasons described elsewhere in the paper.

2.2.1. Surface Plasmon Resonance (SPR)

FcγR/IgG binding analyses were performed on a ProteOn XPR36 SPR Protein Interaction Array System (Bio-Rad Laboratories, Hercules, CA). The ProteOn GLC sensor chips were pre-conditioned with short pulses each (60 s) of 0.5% (w/v) SDS, 50 mM NaOH and 100 mM HCl in both orientations and equilibrated with the experiment buffer, PBSTE (20 mM sodium phosphate, 150 mM NaCl, 0.005% (w/v) polysorbate 20, and 3 mM EDTA, adjusted to pH 7.4). Next, the surface of a GLC sensor chip was activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.2 M) and sulfo-*N*-hydroxysuccinimide (0.05 M; Bio-Rad Laboratories) for 4 min at 30 μL/min. Mouse monoclonal anti-histidine antibody (R&D Systems) prepared in sodium acetate (pH 4.5) was immobilized on the activated channels of the GLC sensor chip to a density of 5000–6000 resonance units (RU) using manufacturer recommended amine coupling procedure. Following the immobilization, the surface was deactivated with 1 M ethanolamine (4 min at 30 μL/min) to quench any unreactive ester groups.

The poly-histidine tagged Fcγ receptors were prepared in PBSTE and captured in the orthogonal orientation of the anti-his antibody immobilization (human and cyno FcγRI: 18 RU, mouse FcγRI: 30 RU, human FcγRIIa: 180 RU, cyno FcγRIIa: 42 RU, human FcγRIIb: 77 RU, cyno FcγRIIb: 35 RU, mouse FcγRIIb: 90 RU, hu FcγRIIIa-V158, cyno and mouse FcγRIII: 18 RU, mouse FcγRIV: 105 RU, all values are average capture levels from different channels). Following the receptor capture, the IgGs were prepared in running buffer at the indicated concentration series and injected across the reference (interspot or uncaptured surface) and the Fcγ receptor captured surfaces at a flow rate of 50 μL/min at varying association and dissociation times for each Fcγ receptor using the one-shot kinetics method (for RI: [IgG] = 500 nM − 6.17 nM at 3-fold dilutions, RII: [IgG] = 8000 nM − 500 nM at 2-fold dilutions, RIII and RIV: [IgG] = 1000 nM − 62.5 nM at 2-fold dilutions, for mouse RIII: [IgG] = 4000 nM − 250 nM at 2-fold dilutions). Following the dissociation phase, the surfaces were regenerated via 15 s pulses of 0.85% (v/v) phosphoric acid in both orientations and short injection of PBSTE (60 s) for equilibration for next cycle of Fcγ/IgG interactions.

Fcγ/IgG interaction sensorgrams were analyzed using the ProteOn Manager Software version 3.0 (Bio-Rad Laboratories). The responses of buffer injection and responses from the interspot channels (or channels without any receptor capture) were subtracted to account for non-specific binding and injection artifacts. Global kinetics analyses of IgG interactions with RI, RIII and RIV were performed using a 1:1 Langmuir binding model and the equilibrium dissociation constant (K_D) was calculated using the ratio of the k_{off}/k_{on}. For weak interactions (RIIa, RIIb, and mouse RIII), binding analyses were performed using equilibrium steady-state model by fitting the steady-state binding response as a function of IgG concentration using nonlinear least-squares optimization to obtain K_D. The values reported are an average of n = 2 or n = 3 replicates.

2.2.2. BioLayer Interferometry (BLI)

BioLayer Interferometry has been used as an alternative label-free method to extract kinetic data for biomolecular interactions [18]. Kinetic analysis of binding of wild type human IgGs to the Fcγ receptors was performed on an Octet RED384 instrument (ForteBIO, Pall Life Sciences). After testing a variety of biosensors and experimental conditions, the assay was performed using the FAB2G sensors in which IgG is immobilized onto human CH1 binding sensor tips [32] in an optimized assay buffer of 1X PBS, 0.05%BSA, 0.01% tween20. For control mouse and cynomolgus monkey IgGs, binding to FAB2G sensors was first tested and optimized. For good curve-fitting and reproducible kinetic results, amount of IgG loaded onto the biosensor was experimentally optimized for each receptor/IgG combination (10–45 μg/ml, for 5–10 min). During the association step, 2-fold serial dilution series of six or seven concentrations of the FcγRs, with the highest at ~$10*K_D$ (known or predicted), along with buffer-only wells were used. For low affinity interactions, $10*K_D$ will be too high that could introduce artefactual results. So, highest FcγR concentration was empirically determined. Sensor tips were equilibrated in assay buffer prior to experiment as well as prior to association (to establish baseline) and during dissociation steps. Experiments were performed at 30 °C with shaking and for weak affinity interactions high sensitivity kinetic acquisition mode was used. Data was analyzed using the Octet Data Analysis software. For high affinity IgG-receptor interactions 1:1 Langmuir Binding Model was used to calculate binding affinity. For weak affinity interactions, Steady-State equilibrium analysis was used to estimate binding affinities.

For validation, the assay was also performed by reversing orientation in which receptor was immobilized using Ni-NTA biosensors and a dilution series of test IgG used in the association step (data not shown). Calculated binding affinities were in good agreement to FAB2G sensor based experiments.

3. Results

Data presented in this section and following discussion is primarily from Surface Plasmon Resonance (SPR) studies. Although BLI data are comparable to SPR results and are presented in the supplementary section, one set of data is used for analysis.

3.1. Validation of in-house Fcγ receptor reagents

To validate in-house purified Fcγ receptor reagents and avoid reagent related errors, all SPR interaction assays with the in-house reagents were performed along with corresponding commercial FcγR reagents (Table 1 and Supplementary Table 2). Head-to-head comparison of calculated binding affinities as well as sensorgram profiles shows that in-house reagents behaved very similarly to commercial reagents. Subsequent analyses and discussions are based on data using in-house reagents.

3.2. The high affinity Fcγ receptor: FcγRI (CD64)

FcγRI, referred to as CD64, is unique in at least two key points from the rest of the Fcγ receptors. First, it is the only high-affinity receptor. For instance, the human FcγRI is a high affinity receptor that binds to human IgG1, IgG3 and IgG4 with nanomolar affinity. Interestingly, human FcγRI has not been shown to bind to human IgG2. Secondly, FcγRI has three extracellular Ig-like domains, unlike the other receptors that have only two. Although there are several studies, including at least three high-resolution structures of Fc complexes [33–35], there is no consensus on the functional importance of the additional domain or the reason for the high affinity.

SPR sensorgrams of IgG binding to FcγRIs are shown in Fig. 1 and binding affinities are shown in Table 2. BLI data yielded similar affinity values and is included in Supplementary Fig. 2. Sensorgrams as well as calculated affinities, expressed as dissociation constant values (K_D), revealed interesting insights. First, binding behavior as well as affinity of mouse FcγRI to wild type human IgGs was different from FcγRIs of the other two species. Mouse FcγRI bound to human IgG1 or human IgG4 with significantly weaker affinity with fast on and off rates. This has been observed in other studies [1,27] and is expected considering the sequence divergence of mouse FcγRI (Supplementary Fig. 1).

Another observation was that, although human and cyno FcγRIs share more than 95% sequence identity in their ECDs (Supplementary Fig. 1), the cyno FcγRI had a relatively higher affinity to all human IgG subclasses than human FcγRI. For instance, human IgG2 showed weak binding to cyno FcγRI while no measurable binding was observed for

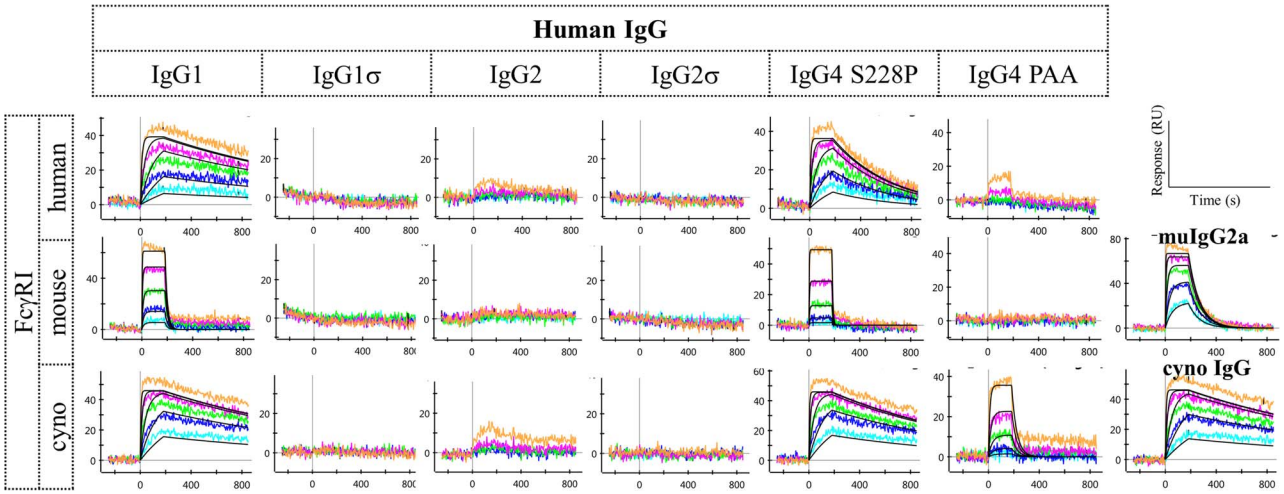

Fig. 1. SPR sensorgrams of wild type and silent human IgG subclasses binding to human, mouse and cynomolgus monkey FcγRIs. K_D values were calculated using the 1:1 Langmuir binding model and are shown in Table 2. [IgG] = 6.17 nM − 500 nM at 3-fold dilutions.

mouse and human FcγRIs. Also, cyno FcγRI showed slightly higher affinity towards human IgG1 and IgG4 than human FcγRI (Figs. 1 and 4, Table 2). IgG1σ and IgG2σ were truly silent without detectable binding to any of the FcγRIs. However, human IgG4 PAA interacted quite strongly with cyno FcγRI and weakly to human FcγRI while no measurable interaction was seen with mouse FcγRI (Table 2, Fig. 1).

3.3. The inhibitory Fcγ receptor: FcγRIIb (CD32b)

The inhibitory receptor FcγRIIb, also referred to as CD32b, is expressed in many immune effector cells and play key roles in regulating activities of activating Fcγ receptors [36]. Moreover, FcγRIIb is the only inhibitory Fcγ receptor known. Likely due to these, this inhibitory receptor is conserved throughout evolution. So, this receptor is present in humans as well as in mouse and cynomolgus monkey.

The inhibitory Fcγ receptor FcγRIIb is a low affinity receptor. Consistent with previous observations, binding affinities for FcγRIIbs from the three species in this study against the human IgG subclasses were generally weak (Fig. 2, Table 2). However, there were noteworthy differences. First, cynomolgus monkey FcγRIIb had a relatively stronger affinity to human IgGs in comparison to human and mouse FcγRIIbs. Particularly, human IgG2 exhibited a significantly higher affinity to cynomolgus monkey FcγRIIb than either human or murine FcγRIIb. Interestingly, human and cynomolgus monkey FcγRIIb ECDs share more than 90% sequence identity. This observation necessitates that at least for human IgG2 based therapeutic Abs, this key difference should be seriously considered when designing monkey based safety/efficacy studies.

3.4. Other activating Fcγ receptors

In humans, there are two sets of weaker affinity activating Fcγ receptors: CD32 (FcγRIIa, FcγRIIc) and CD16 (FcγRIIIa, FcγRIIIb) with several polymorphisms having significant differences in their IgG affinities [24,37]. Both mice and NHPs also have two sets of weak/medium affinity activating receptors, orthologous to the human receptors. NHP's have FcγRIIa and FcγRIII, orthologous to corresponding human receptors, with extensive polymorphisms [38]. Mice have a weak affinity FcγRIII, which is orthologous to human FcγRIIa and medium affinity receptor FcγRIV, orthologous to human FcγRIII [16,25].

In this section and following discussion, human polymorphisms FcγRIIa (R131) and FcγRIIIa (V158) are used for comparison to cyno and mouse Fcγ receptors. Residue corresponding to R131 is a histidine in both murine and cyno weak affinity receptors, similar to the slightly higher affinity human FcγRIIa H131 polymorphism. Human FcγRIIIa (V158) polymorphism has generally higher affinity towards human IgGs than the F158 polymorphism [39]. In cynomolgus monkey and mice, residue corresponding to human V158 is either isoleucine (cyno

Table 2
SPR binding affinities (K_D, nM, N = 2 or 3) of human, mouse and cynomolgus monkey FcγRs to α-RSV/human IgG subclasses. Silent subclasses huIgG1σ and huIgG2σ had no measurable binding to any of the Fcγ receptors and are not included here.

	FcγRI, K_D (nM)			FcγRIIb, K_D (nM)		
IgG	Human	Cyno	Mouse	Human	Cyno	Mouse
huIgG1	7.13 ± 3.28	1.76 ± 0.03	74.9 ± 4.4	6170 ± 416	3063 ± 55	10667 ± 808
huIgG2	NB	WB	NB	NB	2650 ± 40	NB
huIgG4	8.62 ± 1.06	1.99 ± 0.20	246 ± 30	6297 ± 284	6813 ± 586	WB
huIgG4PAA	WB	151 ± 50	NB	WB	NB	NB

		Other FcγRs, K_D (nM)				
IgG	huFcγRIIa (R131)	cyFcγRIIa	muFcγRIII	huFcγRIIIa (V158)	cyFcγRIII	muFcγRIV
huIgG1	6793 ± 535	5697 ± 462	8100 ± 737	247 ± 91	232 ± 29	192 ± 37
huIgG2	> 8000	3877 ± 12	NB	NB	NB	NB
huIgG4	7613 ± 570	> 8000	NB	NB	WB	WB
huIgG4PAA	WB	NB	NB	NB	NB	NB

NB- no measurable binding within tested concentrations, WB- weak binding observed but could not reliably calculate affinity value.

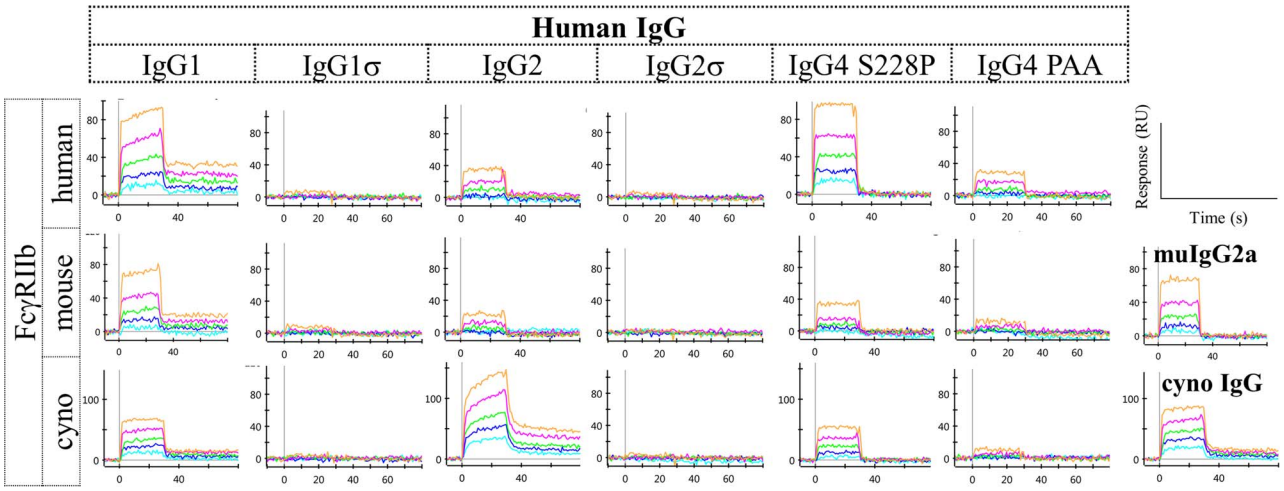

Fig. 2. SPR sensorgrams of wild type and silent human IgG subclasses binding to human, mouse and cynomolgus monkey FcγRIIbs. K_D values were calculated using Steady State equilibrium model and are shown in Table 2.

FcγRIII, mouse FcγRIV) or leucine in mouse FcγRIII (Supplementary Fig. 1).

Among these weak/medium affinity Fcγ receptors from human, mice and cynomolgus monkey, some orthologs exhibit similar binding characteristics toward human IgG subclasses (Fig. 3, Table 2). For instance, human FcγRIIIa (V158), cyno FcγRIII and mouse FcγRIV bind to human IgG1 with affinities in the 200 nM range, in agreement with previous reports [25]. Moreover, no detectable binding was observed for other IgGs for all three of these orthologous receptors, except for a slightly higher signal for cyno FcγRIII-human IgG2 pair. It is however important to note that although their ECDs exhibit similar binding characteristics to human IgGs, functional variations may exist. For instance, cyno FcγRIII has been shown to exhibit divergent functional properties in comparison to its human orthologue, likely due to differences in their intracellular domains [19]. For completeness, sensorgrams for IgG binding to human FcγRIIIa (F158) and FcγRIIIb are given in Supplementary Fig. 2.

Comparing the other orthologous triad (human FcγRIIa, cyno FcγRIIa & mouse FcγRIII), mouse FcγRIII exhibits distinct binding characteristics to human IgGs (Fig. 3). For instance, it showed very weak binding to human IgG1 and no detectable binding to either human IgG2 or IgG4, in contrast to its human and cyno orthologous receptors, which showed weak but detectable binding to both IgG2 and IgG4. Between the human and cyno FcγII receptors however, slightly enhanced affinities for the cyno receptor were observed compared to the human receptor. Particularly, against human IgG2, cyno FcγRIIa bound with low micromolar affinity while human FcγRIIa binds very weakly (Fig. 3). Moreover, cyno FcγRIIa had slightly enhanced affinity to human IgG2 than either human IgG1 or IgG4. Of note is that the cyno FcγRIIa has a histidine at position corresponding to 131 of the human FcγRIIa, which imparts higher affinity for the human receptor.

4. Discussion

Preclinical animal studies are critical steps in drug development. In antibody drug development, mouse and NHP models suitable for the specific disease are usually employed to glean key properties of the molecules prior to human clinical trials. Candidate antibodies are increasingly based on human IgG subclasses and many approaches are pursued to simulate the human system as close as possible. For instance, highly specialized genetically modified mouse models are available for most disease conditions. In other cases, alternating human and NHP antigens are used during phage panning to select for candidate Abs that could cross-react. Despite these and many other methodologies, the translation rate from a successful preclinical study to an FDA approved drug is extremely low (~10%) [40]. Moreover, there were several high-profile mismatches of NHP preclinical and First-in-Human clinical trial results [22,23]. This is attributed likely to lack of detail understanding of model animal biology, particularly IgG/Fc receptor interaction. As a direct result, there has been renewed interest to understand mouse and NHP FcγR/IgG interaction biology [14,17,18,21,41].

In this study, binding characteristics of murine and cynomolgus monkey Fcγ receptors to wild type and Fc-silent human IgG subclasses commonly used in antibody drug development (IgG1, IgG2, IgG4 S228P, IgG1σ, IgG2σ and IgG4 PAA) were systematically compared to selected human Fcγ receptor polymorphisms. Though there are several NHP species used in preclinical studies, cynomolgus monkey is by far the most common in therapeutic antibody drug development, hence the reason for focus in this study. Human IgG3 was not included in this study because it is not a commonly used subclass. Other studies have reported IgG binding affinities of Fcγ receptors of any two species [14,27]. This study compared receptors from the three species to elucidate subtleties among them. It is important to note that for an FcγR/IgG pair, affinity values from *in vitro* label-free methods could significantly differ from study to study or even platform to platform due to variations in experimental conditions, capture method, sensor technology, non-specific binding, or data processing methodology. Moreover, the variable domain of test IgGs can affect binding to receptors. In this study, IgG variable domains were kept identical in test IgGs and binding to human Fcγ receptors and to two model animal receptors were evaluated under identical experimental conditions. Additionally, although two label free methods (SPR and BLI) were used to obtain binding data, analysis and comparisons were made based on one set of data for consistency.

A third label free method (Competition AlphaScreen, Perkin Elmer), was also evaluated as an alternative and significant amount of data was collected. IC50 values from competition AlphaScreen have been used to approximate K_D [42]. However, concentration dependent variations of calculated IC50 s were observed. This may have been a direct result of the assay format used – because the Fcγ receptors are C-terminal multi-His tagged, Ni^{2+} -IMAC beads were the acceptor chromophores. So, data obtained were primarily employed as a ranking tool and not presented here.

Binding affinities are summarized in Table 2 and presented graphically in Fig. 4. Overall, affinities agreed well with published results. In comparison to both human and cyno FcγRs, mouse Fcγ receptors exhibited the most divergent binding properties. This was observed in other studies [27] and was not unexpected since mouse FcγRs showed the most sequence divergence from those of other two species (Supplementary Figure I). Mouse FcγRs showed the weakest affinities to

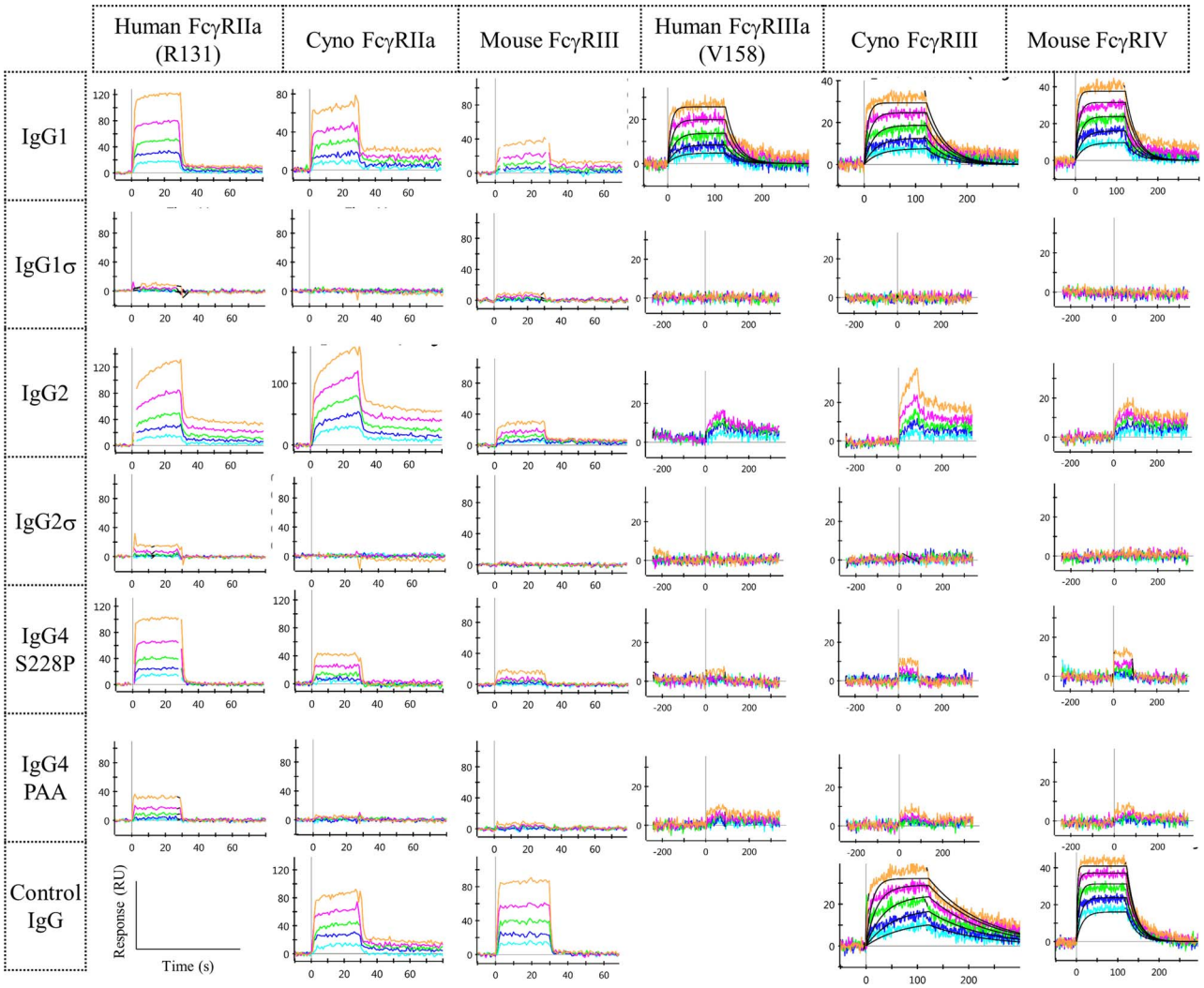

Fig. 3. SPR sensorgrams of wild type and silent human IgG subclasses binding to weak affinity FcγRs of human, mouse and cynomolgus monkey. K_D values were calculated by 1:1 Langmuir or equilibrium Steady State model and are shown in Table 2.

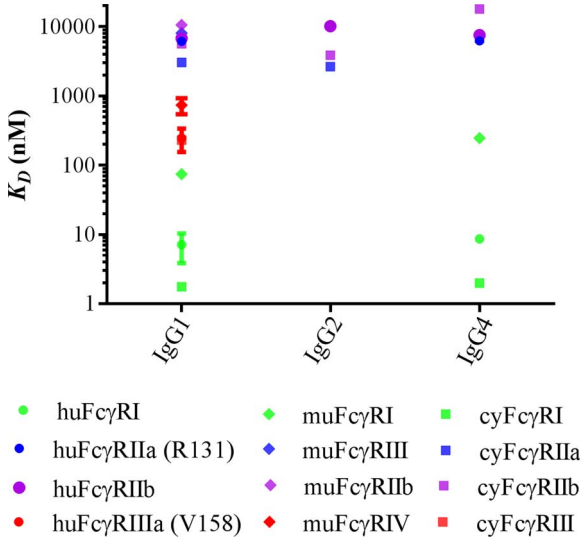

Fig. 4. Graphical representation of affinities of the different Fcγ receptors to human IgG subclasses (SPR data). Weak binders are not represented.

human IgG subclasses, particularly to human IgG2 and IgG4. Between human and cyno receptors, despite sharing very high sequence similarity in their ECDs, important distinctions were observed. Cyno FcγRs showed consistently tighter affinities to human IgGs than human Fcγ receptors.

One of the most striking results of our study was that, while IgG1σ and IgG2σ did not show measurable binding to all receptors, human IgG4 PAA, which is currently pursued for many drug development programs as a Fc-silent framework, bound to cyno FcγRI with nanomolar affinity while its binding to human FcγRI was weak. Although in the *in vivo* (cellular/organismal) context other factors such as cell type, receptor density, activity threshold, and other factors could dictate the functional readout, this *in vitro* data provided the first glimpse of a possibly different response in the test animal.

This enhanced binding was not limited to cyno FcγRI-human IgG4 PAA subclass interaction but extended to other cyno FcγRs-human IgG subclass interactions. For instance, human IgG2 bound more strongly to cyno FcγRs than both human and mouse FcγRs. Both human and mouse FcγRIs did not have measurable binding to the IgG2 subclass while cyno FcγRI had significantly higher binding. This may be due to structural constraints since compared to IgG1 or IgG4, IgG2's lower hinge region is one residue shorter (lacks a glutamate) and a valine replaces leucine.

In support of this, a glutamate to leucine mutation on murine IgG2a resulted in a 100-fold improvement in affinity for human FcγRI [43]. Although human IgG2 has valine at that position, which is not a dramatic change from leucine, the combined effect of absence of glutamate and shortened lower hinge may contribute to its observed affinity to FcγRI. Similarly, cyno FcγRIIb showed a higher affinity to the human IgG2 subclass compared to the other two FcγRs, which was also observed in other studies [14].

It has been observed previously for human FcγRIIa that an H131 polymorphism shows enhanced affinity to human IgG2 compared to the R131 polymorphism [44]. Human FcγRIIb and cyno FcγRIIb have an arginine and a histidine, respectively, at position corresponding to 131 on human FcγRIIa (Supplementary Fig. 1). Affinity of cyno FcγRIIb to human IgG2 however was significantly enhanced, which was a likely result of combination of structural perturbations on the IgG as well as the histidine residue on the receptor binding site. These persistent observations suggested that at least for IgG2 based therapeutic candidate molecules, preclinical test designs in cynomolgus monkeys should take such discrepancies into account.

The V158 polymorphism of human FcγRIIIa was shown to bind slightly tighter than the F158 polymorphism [39]. This may simply be a result of steric hindrance due to the bulky side chain of phenylalanine. This position is isoleucine in both cyno FcγRIII and mouse FcγRIV while it is a leucine in mouse FcγRIII (Supplementary Fig. 1). Although there are IgG subclass specific variations, the affinity of both cyno FcγRIII and mouse FcγRIV to human IgG1, for instance, are comparable to that of human FcγRIIIa (V158) while it is significantly weaker for mouse FcγRIII reinforcing the idea that affinity to IgG Fcs may be sterically influenced by the identity of amino acid at this position.

5. Conclusion

This study provided a side-by-side comparison of binding affinities of Fcγ receptors from two model animals and humans to human IgG subclasses most commonly used in therapeutic antibody development. Of significance was the finding that cyno Fcγ receptors exhibited enhanced affinity to human IgG subclasses despite their sequence similarity to human Fcγ receptors. The observation that a presumably silent IgG4 PAA subclass bound to cyno FcγRI with nanomolar affinity while it interacted weakly with human FcγRI suggested that human IgGs should not be assumed to behave similarly in cynomolgus monkey model animals as they behave in the human system. It also suggests that a detailed in vitro binding study could signal any potential discrepancies and help in preclinical study design, particularly for IgG2 and IgG4 PAA based therapeutic programs. These results will help design better preclinical animal studies in wild type mice or cynomolgus monkey models.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

MGD and MLC conceived study. MGD, RKN and MLC designed experiments. MGD and RKN conducted experiments and analyzed data. MGD wrote paper with MLC. GLG and ERL reviewed data and analysis, critically reviewed manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.imlet.2018.02.006.

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