Polymorphisms and Interspecies Differences of the Activating and Inhibitory Fc γRII of *Macaca nemestrina* Influence the Binding of Human IgG Subclasses


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Polymorphisms and Interspecies Differences of the Activating and Inhibitory FcγRII of Macaca nemestrina Influence the Binding of Human IgG Subclasses


Little is known of the impact of Fc receptor (FcR) polymorphism in macaques on the binding of human (hu)IgG, and nothing is known of this interaction in the pig-tailed macaque (Macaca nemestrina), which is used in preclinical evaluation of vaccines and therapeutic Abs. We defined the sequence and huIgG binding characteristics of the M. nemestrina activating FcγRIIa (mnFcγRIIa) and inhibitory FcγRIIB (mnFcγRIIB) and predicted their structures using the huIgGFc/huFcγRIIa crystal structure. Large differences were observed in the binding of huIgG by mnFcγRIIa and mnFcγRIIB compared with their human FcR counterparts. MnFcγRIIa has markedly impaired binding of huIgG1 and huIgG2 immune complexes compared with huFcγRIIa (HIs131). In contrast, mnFcγRIIB has enhanced binding of huIgG1 and broader specificity, as, unlike huFcγRIIB, it avidly binds IgG2. Mutagenesis and molecular modeling of mnFcγRIIa showed that Pro159 and Tyr160 impair the critical FG loop interaction with huIgG. The enhanced binding of huIgG1 and huIgG2 by mnFcγRIIB was shown to be dependent on HIs131 and Met132. Significantly, both His131 and Met132 are conserved across FcγRIIb of rhesus and cynomolgus macaques. We identified functionally significant polymorphism of mnFcγRIIa wherein proline at position 131, also an important polymorphic site in huFcγRIIa, almost abolished binding of huIgG2 and huIgG1 and reduced binding of huIgG3 compared with mnFcγRIIa His131. These marked interspecies differences in IgG binding between human and macaque FcRs and polymorphisms within species have implications for preclinical evaluation of Abs and vaccines in macaques. The Journal of Immunology, 2014, 192: 792–803.

The fe Fc receptors (FcRs) are cell surface molecules primarily expressed on effector leukocytes of the innate immune system and by binding Igs provide adaptive immunity with a cell-based effector system (1). There are three classes of leukocyte IgG FcRs in humans: the high-affinity IgG receptor FcγRI (CD64) and the low-affinity IgG receptors FcγRII (CD32) and FcγRIII (CD16).

The two major genes of the human (hu)FcγRII family encode FcγRIIa and FcγRIIB and their splice variants. These receptors play activating and inhibitory roles in normal immune responses and immune homeostasis (1, 2), and imbalance in these opposing roles is a key contributing factor to the development of pathological inflammation in several autoimmune diseases (3). FcγRIIa is one of several activating FcRs, but it is the most widespread and abundant FcγR of humans present on all leukocytes except lymphocytes. Despite being a low-affinity receptor, FcγRIIa avidly binds oligovalent Ab-coated targets (immune complexes) to induce cytokine release from inflammatory leukocytes, respiratory burst, Ab-dependent cell-mediated killing, internalization of complexes, and platelet aggregation. FcγRIIb in humans is a powerful inhibitor of immune receptor signaling and is critical to the modulation of humoral immunity and Ab-dependent immune functions (4–6). Its ITIM-dependent modulation of ITAM signaling cascades regulates B cell Ag receptor signaling and consequent Ab responses. FcγRIIb also regulates signaling by the activating FcRs FcγRI, FcγRIIa, FcγRIIIa, FcεRI, and FcαRI. In a practical sense, the IgG–FcγR interaction is a key contributor to the effectiveness of many vaccines both at the level of immune regulation and induction of effector function. Indeed, it has been suggested that the IgG–FcγR interaction mediating Ab-dependent cell-mediated cytotoxicity may play a role in HIV vaccine–induced protective immunity of humans (7). Moreover, the effectiveness of allergen immunotherapy (8) and many therapeutic mAbs, particularly anticancer mAbs, has been attributed at least in part to successful engagement of FcR-dependent effector systems, including FcγRIIa and FcγRIIb (1).

In humans, several polymorphisms of the activating IgG receptor, FcγRIIa, are known (9, 10). The most clinically significant polymorphism encodes amino acid position 131 where either a histidine or an arginine residue may be present, resulting in profound effects on binding of huIgG2 (11).
Genetic polymorphisms of huFcRs that affect their capacity to bind IgG or alter the balance of activation over inhibition have been implicated in resistance to HIV and bacterial infection (12–18), susceptibility to autoimmunity (19, 20), and the effectiveness of therapeutic mAbs, mostly IgG1 but increasingly IgG2 (1). As a result of the success of mAbs, considerable effort has been made to improve their FcR-dependent potency by engineering Fc portions for the purpose of selective engagement of either activating FcRs (1), including FcγRIIa (21) or inhibitory FcγRIIB (22). Furthermore, the most clinically significant polymorphism of the activating IgG receptor, FcγRIIa (9, 10), encodes either a histidine or an arginine residue at amino acid position 131 and influences the clinical outcome of Ab therapy (23) and additionally has profound effects on binding of huIgG2 (11).

The genetic diversity of nonhuman primate (NHP) FcRs has not been extensively characterized, even though NHPs are key animal models for many diseases, including HIV. Examination of functional Fc polymorphisms in NHPs is, as found in humans, pertinent to understanding susceptibility to infectious and autoimmune diseases. Interspecies functional substitutions will likely substantially influence the evaluation of human Abs in NHPs.

Indeed, although evolutionarily conserved, the limited information available to date shows that significant interspecies sequence differences are apparent between the huFcRs and their orthologs in different NHP species (24–27). Some of this sequence diversity occurs at sites that are predicted by homology to be essential for the interaction with IgG and may influence the binding of IgG. These differences may result in alterations to the relative contributions that the different FcR classes make to Ab-dependent effector function in vivo in macaques compared with humans. Correspondingly, differences in species IgG–FcR interactions may greatly complicate the interpretation of preclinical studies aimed at evaluating the functional activity of therapeutic Abs or vaccines in NHP models such as macaques, including Macaca nemestrina. Additionally, use of outbred populations of macaques may further complicate interpretation owing to nonsynonymous polymorphisms in the macaque FcR genes.

Little is yet known of the impact of FcR polymorphism on the binding of huIgG in NHPs, especially in the three macaque species commonly used in medical research: rhesus (Macaca mulatta), cynomolgus (Macaca fascicularis), and pig-tailed (M. nemestrina) macaques. Furthermore, nothing is known of the activity of FcRs of the pig-tailed macaque. Similar to cynomolgus and rhesus macaques (28, 29), pig-tailed macaques are used in the evaluation of Ab immunotherapy (30–32), humoral immune responses to infection, and vaccine candidates, including dengue virus (33, 34) and HIV/simian HIV (35, 36). Furthermore, different species have been used in similar models (30, 32).

In this study, we define the ligand-binding properties of activating FcγRIIa and the inhibitory FcγRIIb of M. nemestrina (mnFcγRIIa and mnFcγRIIb). We identify a functionally significant polymorphism of FcγRIId and define the molecular and structural basis of impaired binding to IgG by mnFcγRIIa and for the enhanced binding and broader specificity of mnFcγRIIb compared with their human orthologs (huFcγRIIa and huFcγRIIb). These data also have implications for the analysis of IgG function in other macaque species and NHPs.

**Materials and Methods**

**Animals**

Outbred 3- to 5-y-old pig-tailed macaques (M. nemestrina) were obtained from the Australian National Macaque Breeding Facility, and studies were approved by the University of Melbourne and Commonwealth Scientific and Industrial Research Organization Animal Health Institutional Animal Ethics Committees. Whole venous blood was obtained from animals sedated with ketamine as previously described (36), and PBMCs were isolated over Ficoll-Hypaque and stored in liquid nitrogen.

**Cloning of FcγRs**

Gene transcripts for mnFcγRIIa and mnFcγRIIb were obtained by PCR of cDNA (AffinityScript quantitative PCR cDNA synthesis kit from Agilent Technologies, Santa Clara, CA) produced from total RNA (RNeasy Mini Kit from Qiagen, Melbourne, VIC, Australia) from PBMCs of unrelated animals. Restriction enzymes and DNA-modifying enzymes were all from New England Biolabs (Beverly, MA), except for PCR applications, which used AccuPrime Pfx DNA polymerase (Life Technologies, Melbourne, VIC, Australia). The primers to generate FcγR PCR fragments were synthesized by Sigma-Aldrich (Sydney, NSW, Australia).

Because of the absence of any DNA sequence information from M. nemestrina, the 5'- and 3'-amplifying primers for FcγRIIa or FcγRIIb were based on sequences of rhesus macaque (M. mulatta, GenBank NM_001257300) or cynomolgus macaque (M. fascicularis, GenBank AF485141.1), respectively. The FcγRIIa 5’ primer was complementary to the initiation codon and the following five codons, and the 3’ primer was complementary to the last seven codons and the termination codon. The FcγRIIb 5’ primer was complementary to the initiation codon and the first five codons, and the 3’ primer was complementary to the final six codons and the termination codon. The primers also contained KpnI and EcoRV sites. The Kpn1/EcoRV digested PCR products were cloned into PENTR1A (Life Technologies) followed by Gateway LR cloning (Life Technologies) into a Gateway-adapted pMXI expression vector containing a neomycin resistance cassette (37).

The huFcγRIIb2 construct was obtained from PBMC-derived cDNA as above using 5’ and 3’ primers based on the sequence of FcγRIIb (38) and cloned into pMXI as above.

The nucleotide sequences of mnFcγR were determined from both PBMC-derived PCR-amplified cDNA and from six FcγR clones for each receptor from each individual animal using BigDye version 3.1 terminator cycle sequencing (Applied Biosystems, Melbourne, VIC, Australia) and separated at the Australian Genome Research Facility (Melbourne, VIC, Australia). All cDNA sequences have been submitted to GenBank (submission nos. 1637005 and 1655933).

Alignments of FcγRII sequences were generated using CLC Sequence Viewer version 6.4 (CLC bio, Aarhus, Denmark).

Isolation and expression of the human FcγRIIa-H131 and FcγRIIa-R131 have been described previously (39–41).

**Site-directed mutagenesis**

Mutated FcγRs were constructed by in vitro site-directed mutagenesis using a series of mutation primers and the thermostable polymerase Pfx (Life Technologies) to obtain the following for mnFcγRIIa-allele 1 (mutant 1, N135T [N→T]; mutant 2, P139L, Y160F [PY→LF]), mnFcγRIIa-allele 2 (mutant 3, P131H, M132L, N133D [PMN→HLD]), the double mutants of mnFcγRIIa-allele 2 (mutant 4, PMN→HLD with N→T; mutant 5, PMN→HLD with PY→LF), and for mnFcγRI Ib1 (mutant 6, H131R, M132S [HM→RS]).

**Stable expression of FcγRII**

A pMXI retroviral expression system was used to introduce FcγR DNA into the FcR-deficient IIA1.6 cells. Phoenix packaging cells were maintained in RPMI 1640 containing glutamine and 5% heat-inactivated fetal bovine serum and were transfected with FcγRII plasmids using Lipofectamine 2000 (Life Technologies) to generate retrovirus. The retroviral suspension was overlaid onto IIA1.6 cells as described (39). Transduced IIA1.6 cells were selected for resistance to 0.4 mg/ml Geneticin (Life Technologies).

**Abs**

Purified human myeloma proteins IgG1k, IgG2a, and IgG3e were purchased from both Sigma-Aldrich (Castle Hill, NSW, Australia) and The Binding Site (Birmingham, United Kingdom). PE-conjugated F(ab’)2 goat anti-human IgG F(ab’)2-specific polyclonal was from Jackson Immuno-Research Laboratories (West Grove, PA). Human IgG in the form of i.v. IgG was obtained from Novartis (Sydney, NSW, Australia). Mouse IgG was obtained from Autogen Bioclear (clone L27; BD Biosciences, Sydney, NSW, Australia), anti-CD14 (clone m52; BD Biosciences), anti-CD56 (clone NCAM162; BD Biosciences), anti-CD159a (NKG2) (clone Z199; Beckman Coulter, Melbourne, VIC, Australia), anti-FcγRII (clone 3G8; BD Biosciences), and anti-CD41a (clone HIPS; BD Biosciences).
The anti-FcγRIIA mAbs 8.7, 8.2, and IV3 and the anti-FcγRIIB monocl- onal X63-21/7.2 have been previously described (40, 42, 43). Fab fragments of IV-3 and Fab(β)2 fragments of 8.7, 8.2, and X63 mAbs were generated as described (42) and then biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL).

IgG ligands

Two separate methods were used to evaluate the binding of huIgG to cell surface–expressed FcRs of M. nemestrina and humans. First, for anti-Fab complexes, complexes of IgG were generated by cross-linking IgG with Fab(β)2 fragments of fluorochrome-conjugated anti-human Fab as previously described (44). Briefly, individual human IgG1, IgG2, or IgG3 subclasses were incubated with Fab(β)2 fragments of PE-conjugated goat anti-human IgG Fab(β)2 at a 2:1 ratio for 30 min at 37°C and then on ice for 5 min. Second, for Fcγ dimers/trimers, biotinylated IgG dimmer/trimer complexes of pooled huIgG were generated using the cross-linker Tris-succinimidylaminomethylamine ( thermo Scientific). Tris-succinimidylaminomethylamine at 5 mg in 0.5 ml anhydrous DMSO was mixed with 3.85 mg biotin-X-hydrazide (Sigma-Aldrich) in 0.5 ml anhydrous DMSO for 2 h at room temperature to generate the intermediate biotinyl bis-succinimidyl amidotriacetate, which was used without purification. Human IgG4 (4 ml at 25 mg/ml in PBS) was mixed with an 11-fold excess of biotinyl bis-succinimidylaminomethylamine (0.7 ml) and allowed to react for 1 h at room temperature. The resulting IgG dimers/trimers were purified from the reaction mixture and separated from monomers and multimers by gel filtration on a Sephacryl S-300 column (1.5 × 100 cm). Fractions were analyzed by SDS-PAGE, and those corresponding to IgG dimers/trimers were pooled and stored in aliquots at −80°C.

Flow cytometric detection of immune complex binding and receptor expression

The binding of IgG complexes to allelic forms of receptors and mutants thereof was determined as described (40). Briefly, anti-F(ab′)2 PE/IgG complexes at the indicated concentrations were incubated with 1.2 × 10⁶ cells in 50 μl for 1 h on ice and then washed twice and resuspended in 200 μl PBS/0.5% BSA. Similarly, aggregated huIgG or biotinylated IgG dimer/trimer binding was detected by indirect fluorescence following incubation with cells on ice for 1 h. Cells were then washed and incubated with PE-conjugated goat anti-human Ab or allophycocyanin-streptavidin, respectively, for 30 min on ice, washed twice, and then resuspended in 200 μl PBS/BSA. Background binding controls included nonspecific binding of IgG to untransfected IIA1.6 cells and nonspecific binding of conjugate only to FcR-expressing cells.

Expression levels of FcγR on the transfected cells were determined in each experiment by flow cytometry using the following biotinylated anti-peptide mAbs: clone 8.7, 8.2, and Fab(β)2 for the anti-FcγRIIB X63- 21/7.2 (F(ab′)2) (40). Cells were then washed and binding was detected using allophycocyanin-streptavidin as described above. Gating was set to record fluorescence of 10,000 viable single cells. The analysis of huIgG binding to each human or macaque FcγR and mutants thereof was performed on at least five independent occasions using the anti-Fab complexes and at least three independent occasions using the IgG dimers/trimers.

Flow cytometric analysis of Fc receptor expression on blood cells

Whole blood was collected from four M. nemestrina as above and from healthy human volunteers after informed consent as approved by the Alfred Health Human Ethics Committee or the Monash University Standing Committee on Ethics in Research Involving Humans. PBMCs were isolated on a Ficoll density gradient. Total leukocytes were obtained from buffy coats and then erythrocytes were lysed with hypotonic RBC lysis solution (Miltenyi Biotec, Sydney, NSW, Australia) according to the manufacturer’s instructions. Expression of surface markers was determined using flow cytometry where human and M. nemestrina B lymphocytes were identified with anti-CD20 and monoclones were identified with anti-CD14. Human NK cells were defined with anti-CD56 and macaque NK cells with anti-CD159a (NK2G) (45). Neutrophils were identified by forward and side scatter profiles. Platelet-rich plasma was isolated by low-speed centrifugation and platelets were identified by expression of CD41a. The expression of FcγRIIIa was defined using clone 3G8, and FcγRIII was identified with mAb 8.7 as described above.

Homology modeling of the macaque FcγRIIa/huIgG1-Fc interaction

Protein homology models were prepared using the Discovery Studio suite, version 3.0 (Accelrys, San Diego, CA). The cocrystal structure of huFcγRIIA/ huIgG1-Fc ( Brookhaven Protein Data Bank ID 3RY6) (40) was used as a template to generate a homology model of the macaque FcγRIIa/huIgG1-Fc complex using the amino acid sequence of mnFcγRIIa-allele 1 (Fig. 1). The three-dimensional model of the macaque FcγRIIa/huIgG1-Fc complex was optimized by conjugate-gradient energy minimization against spatial restraints extracted from the 3RY6 template and a probability density function using the Modeller algorithm (46). The protein interface between FcγRIIa and huIgG1-Fc was analyzed using the protein interfaces, surfaces, and assemblies (PISA) server at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (47). The buried surface area of residues at the interface of FcγRIIa and the bound huIgG1-Fc were plotted for comparison with the total buried surface area between huFcγRIIa and mnFcγRIIa-allele 1. Because mnFcγRIIa-allele 1 has one additional N-linked glycosylation site compared with huFcγRIIa, in silico glycosylation of this extra site was performed using the GlyProt Web-based server (http://www.glycosciences.de/modeling/glyprot/php/main.php) (48).

Results

Sequence comparison of huFcγRIIa and mnFcγRIIa

The mnFcγRIIa sequences were determined from cDNA isolated from 10 animals of an outbred colony of M. nemestrina. Replicate sequence analysis of PCR products and of multiple clones from independently derived, duplicate PBMC samples were used to establish and then confirm FcR sequences.

Sequence analysis comparing M. nemestrina sequences to the huFcγRIIa revealed amino acid differences between macaque FcγRIIa and humans that were conserved in all animals and also identified polymorphic sequence variation between the FcγRIIa of individual macaques (Fig. 1). Thus, 26 aa that differed from huFcγRIIa were conserved in all individuals. Sixteen of these amino acid differences mapped to the extracellular region, three to the transmembrane region, and seven to the cytoplasmic tail of mnFcγRIIa (Fig. 1).

Further sequence analysis among the 10 animals revealed extensive polymorphism and identified eight allelic products of M. nemestrina FcγRIIa (Fig. 1). These allelic forms were distinguished from each other by 16 polymorphic residues, of which 9 were located in the extracellular domains (at positions 1, 74, 79, 89, 93, 119, 131, 133, and 140), 1 was located in the transmembrane region, and 6 were found in the cytoplasmic tail of mnFcγRIIa (Fig. 1).

In M. nemestrina, the inhibitory FcγRIIB has 23 nonpolymorphic amino acid differences from huFcγRIIB, and 21 of these occur in the extracellular domains. A single polymorphism resulting in amino acid substitution methionine/valine at position 254 was apparent exclusively from each other by 16 polymorphic residues, of which 9 were located in the extracellular domains (at positions 1, 74, 79, 89, 93, 119, 131, 133, and 140), 1 was located in the transmembrane region, and 6 were found in the cytoplasmic tail. Interestingly, the identity of the nine polymorphic amino acids within the extracellular region suggests that FcγRIIa alleles 1, 3, 4, 6, 7, and 8 are closely related, as they contain the human-equivalent residue in many of the polymorphic positions. In contrast, FcγRIIa alleles 2 and 5, found in 2 of the 10 animals investigated, are closely related to each other but are the most different to huFcγRIIa, with seven or eight of the nine polymorphic positions, respectively, being distinct from huFcγRIIa (Fig. 1).

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Binding of huIgG to mnFcγRIIa

A comparative analysis of the binding of huIgG subclasses to allelic forms of mnFcγRIIa and to huFcγRIIa was undertaken in transfected cells where their equal expression was confirmed by binding of the anti-FcγRIIB mAb F(ab′)2 fragments (Fig. 3A), which detects an epitope in the extracellular domains in FcγRIIa and FcγRIIIa (40).

The binding of huIgG3 complexes to mnFcγRIIa-allele 1 and huFcγRIIa-His131 was analyzed by flow cytometry and was shown to be essentially identical with relative cell surface staining of mean fluorescence intensity (MFI) of 70,000 and 71,000, respectively (Fig. 3D). In contrast, mnFcγRIIa-allele 1 bound IgG1
or IgG2 complexes with MFIs of 5000 and 360, respectively, which were 8- to 10-fold lower than their binding to huFc γRIIa-
-His131 (Fig. 3B, 3C). Thus, in comparison with huFc γRIIa, the mnFc γRIIa-allele 1 has equivalent binding of complexes of huIgG3, but greatly diminished binding of complexes of huIgG1 and huIgG2.

In addition to the differences in huIgG binding between human and M. nemestrina FcyRIIa observed above, even greater differences were found in huIgG binding to the allelic forms of mnFc γRIIa (Fig. 4). The binding of all huIgG subclasses to mnFc γRIIa-allele 2 receptor was reduced greatly (Fig. 4B–D). Compared to binding by mnFc γRIIa-allele 1 (MFI of 5000), IgG1 binding to mnFc γRIIa-allele 2 was reduced 80-fold (MFI of 90) and was barely detectable above background (MFI of 30, Fig. 4B). Similarly, IgG2 binding of mnFc γRIIa-allele 2 was also barely detectable above background levels (Fig. 4C). The binding of IgG3 complexes (MFI of 11,600) was also reduced 6-fold compared with mnFc γRIIa-allele 1 (MFI of 70,300). Thus, not only do interspecies differences of FcR affect huIgG binding, but polymorphisms (intraspaces differences) in mnFc γRIIa also influence the binding of huIgG. The mnFc γRIIa-allele 2 is greatly impaired in ligand binding of human IgG1, IgG2, and IgG3.

Next, we defined the sequence substitutions responsible for the low ligand binding activity of mnFc γRIIa-allele 2. Analysis of the polymorphic amino acid differences in the mnFc γRIIa in the context of known functional regions of huFc γRIIa showed that position 131
is part of the major contact surface in the huFcRgIIa/ IgG interaction (40, 49, 50). Moreover, in humans, polymorphic FcRgIIa (high and low responder allelic products that differ at position 131) have very different interactions with mouse IgG1 and huIgG2 (44). Thus, it seemed possible that the large differences in IgG binding between the two M. nemestrina allelic receptors results from the sequence differences in this segment, namely, HMD(131–133) of the functional receptor encoded by mnFcRgIIa-allele 1 compared with the PMN(131–133) of the poorly functional receptor encoded by mnFcRgIIa-allele 2 (Fig. 1).

Consequently, we attempted to reconstitute binding of huIgG by replacing PMN(131–133) of mnFcRgIIa-allele 2 with HLD(131–133) from huFcRgIIa. Note that His131 and Asp133 of mnFcRgIIa-allele 1 are identical in huFcRgIIa (Fig. 1). Weak binding of IgG1 and IgG2 to mnFcRgIIa-allele 2 was demonstrated (Fig. 4B, 4C), and the HLD(131–133) substitution substantially rescued binding with an MFI of 2900 and an MFI of 300 for IgG1 and IgG2, respectively (Fig. 4F, 4G; blue filled, dashed line) that was comparable to that of mnFcRgIIa-allele 1 (MFI of 5000 for IgG1 and 360 for IgG2) (Fig. 4B, 4C; red filled, dashed line). Similarly, the binding of IgG3 was also improved from an MFI of 11,600 to an MFI of 53,400 (Fig. 4D, 4H). However, this increased binding of IgG was still markedly lower than the binding of IgG1 and IgG2 complexes to the huFcRgIIa-His131; that is, MFIs of 57,200 and 3,600, respectively. Binding of IgG3 by the mnFcRgIIa-allele 2 with HLD(131–133) remained slightly reduced compared with the human receptor (MFI of 53,400 for mnFcRgIIa-His131 compared with 70,100 for huFcRgIIa-His131; compare with Fig. 3). The failure to fully enable IgG binding to levels observed for the human receptor implied that other amino acid residue differences between mnFcRgIIa and huFcRgIIa contribute to ligand binding. Of particular interest in this context were residues Pro159 and Tyr160 in mnFcRgIIa and Leu159 and Phe160 in huFcRgIIa, which are located in the G strand of the second domain adjacent to the critical FG loop that contacts IgG.

Thus, allele 2–encoded mnFcRgIIa, which we had mutated to contain the HLD(131–133) of huFcRgIIa-His131 (Fig. 4E–H, dashed line), was further modified by replacing Pro159 and Tyr160 with the equivalent Leu159 and Phe160 from huFcRgIIa (Fig. 4E–H). This substitution increased IgG1 binding 5-fold to levels similar to huFcRgIIa-His131 and increased the binding of IgG2 almost 10-fold. Interestingly, there was no further improvement in the IgG3 binding beyond that already achieved by the substitution of PMN(131–133) for HLD(131–133).

The role of Pro159 and Tyr160 in macaque receptor binding of IgG1 and IgG2 was further examined in a similar analysis of the mnFcRgIIa-allele 1, which contained the HMD(131–133) sequence. The replacement of Pro159 and Tyr160 with Leu159 and Phe160 from huFcRgIIa also improved IgG1 and IgG2 binding to levels similar to those of huFcRgIIa binding (Fig. 4I–L). Thus, the presence of

FIGURE 2. Alignment of the allotypic form of FcγR IIb of M. nemestrina with the splice variants of its human counterpart (huFcγR IIb1, GenBank NM_001002275.2; huFcγR IIb2, GenBank NM_001002273.2) (38). Amino acids identical to M. nemestrina are shown as dots, and positions of polymorphism are indicated by inverted triangles; the transmembrane region is shown as underlined dashed line, and the deletions within the cytoplasmic tail of FcγR IIb2 resulting from mRNA splicing or within the membrane stalk are indicated by solid lines. Critical residues analyzed in this study are boxed. Trp87 and Trp110 are indicated with stars. M. nemestrina sequences are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank) as follows: mnFcγR IIb1, accession no. KF234403; and mnFcγR IIb2, accession no. KF234404.

FIGURE 3. Comparative binding of huIgG subclasses to mnFcγR IIa-allele 1 (red filled histogram with dashed red line) or the huFcγR IIa-His131 (open histogram, solid red line). Cell surface expression of receptor protein was determined using the anti-FcγR mAb 8.7, which recognizes huFcγR IIa and mnFcγR IIa. Background binding is shown as a solid black line. The binding of each IgG subclass to each receptor was tested on at least five occasions.
His131 in dictating the binding of IgG2. (Fig. 5C), which is entirely consistent with the essential role of

Principal sequence differences occur around position 131 wherein PY(159–160) was replaced by human sequence HLD(131–133) (open histogram, solid blue line). Binding of anti-receptor mAb 8.7 (I) or IgG (J–L) to mnFcRIIa-allele 1 (red filled histogram, dashed line) as in (B–D)) or mnFcRIIa-allele 1 LF where PY (159–160) was replaced with human LF(159–160) (open histogram, solid red line). The binding of each IgG subclass to each allelic receptor was tested on at least five occasions, and the binding to mutated receptors was tested on at least three occasions.

PY(159–160) in mnFcRIIa results in a significant impairment of binding of the human IgG1 and IgG2 subclasses.

Binding of huIgG to mnFcγRIIb

The interaction of huIgG subclasses with the inhibitory FcγRIIb of M. nemestrina was investigated and substantial differences were observed in specificity and binding compared with huFcγRIIb (Fig. 5). Indeed, unlike the activating FcγRIIa of M. nemestrina, the mnFcγRIIb had a 10-fold greater capacity to bind IgG1 complexes than did huFcγRIIb (Fig. 5B). The greatest functional divergence was in the binding of huIgG2, which failed to bind to huFcγRIIb as expected, but surprisingly was strongly bound by mnFcγRIIb (Fig. 5C). FcγRIIb from both species bound IgG3 at similar levels (Fig. 5D).

This difference in the capacity of the inhibitory mnFcγRIIb to bind IgG2 was surprising because in humans IgG2 essentially binds only to the activating FcγRIIa-His131, which is the consequence of the Arg/His131 polymorphism dictating specificity for IgG2 in this activating receptor. Comparison of the amino acid sequence of the IgG binding regions of human and M. nemestrina FcγRIIb shows that the principal sequence differences occur around position 131 wherein mnFcγRIIb encodes His131 and Met132, whereas huFcγRIIb has Arg131 and Ser132 (Fig. 2). Thus, we replaced the HM(131–132) of mnFcγRIIb with the RS(131–132) of huFcγRIIb and measured the impact on IgG1 and IgG2 binding (Fig. 5B, 5C). The substitution of the human-derived RS(131–132) into mnFcγRIIb resulted in a marked loss in IgG1 binding, decreasing to levels equivalent to huFcγRIIb (Fig. 5B). Moreover, IgG2 binding was completely lost (Fig. 5C), which is entirely consistent with the essential role of His131 in dictating the binding of IgG2.

Binding of huIgG dimers to FcγRII

The binding measurements above were obtained using complexes generated by oligomerization of IgG with an anti-Fab Ab, a technique commonly used for the detection of immune complex binding to low-affinity receptors (44). To exclude the possibility that the anti-Fab Ab may influence FcR binding of the IgG complexes, we used a second method where dimers/trimers of huIgG were generated by cross-linking pooled human i.v. Ig with a biotinylated cross-linker (Fig. 6).

Flow cytometry analysis of the binding of the cross-linked IgG dimers/trimers to FcγRIIa and FcγRIIb and receptor mutants entirely agreed with the binding observed using the complexes generated with the anti-Fab Ab (Figs. 3–5). The mnFcγRIIa-allele 2 failed to bind the IgG dimers/trimers, but the replacement of PMN(131–133) with HLD(131–133) enabled binding similar to that of allele 1 (Figs 6A, 6B). Further mutation of this construct wherein PY(159–160) was replaced with the LF(159–160) of huFcγRIIa resulted in further increase of IgG binding to levels approaching those of huFcγRIIa-His131 (Fig. 6A).

One other significant structural difference between humans and macaques in this region is a potential N-glycosylation site at position 135 in mnFcγRIIa, which is absent from huFcγRIIa but is found in both human and macaque FcγRIIb. Because this site sits adjacent to the critical His131, we replaced N135 in mnFcγRIIa with the human equivalent T135; however, this mutation had little effect on the binding of any huIgG subclass (Fig. 6A).

Similarly, the same modifications of mnFcγRIIa-allele 1 (Fig. 6B) were made and resulted in similar effects on IgG binding. Replacement of PY(159–160) with the human LF(159–160) residues greatly improved IgG binding, but as seen in mnFcγRIIa-allele 2, the removal of the 135 glycosylation site had little effect.
In the case of FcγRIIb (Fig. 6C), as was observed with the anti-Fab Ab complexes, the *M. nemestrina* receptors bound IgG dimers/trimers more strongly than did huFcγRIIb, and, as expected, the substitution of the mnFcγRIIa sequence HM(131–132) with the human equivalent RS(131–132) profoundly diminished binding to levels, which were even lower than observed with huFcγRIIb.

**Molecular modeling of mnFcγRIIa**

The human FcγRIIa/IgG interaction has been well characterized by extensive mutagenesis (42, 51, 52), x-ray structure studies of huFcγRIIa alone (50) and in complex with human IgG1-Fc (40), which have collectively identified four structurally contiguous regions that form the IgG binding surface in the second domain of FcγRIIa. To understand the key interspecies sequence differences between macaque and human receptors, we generated a molecular model of mnFcγRIIa-allele 1 interaction with huIgG1 using the x-ray structure of the huFcγRIIa/huIgG1-Fc complex (40). This model (Fig. 7) predicts that key contacts defined in the huFcγRIIa/huIgG1-Fc interface are altered in the interaction of mnFcγRIIa and huIgG.

The FG loop of the second domain of FcγRIIa is one major contact between receptor and IgG-Fc where Tyr^{157} is conserved in all macaque species and humans, makes critical contacts with Leu^{234} and Gly^{236} in the lower hinge sequence LLGG(234–237) on the IgG-Fc B chain (Fig. 7A). Compared to the huFcγRIIa/Fc complex, the contacts between the Tyr^{157} of mnFcγRIIa FG loop and the IgG-Fc are reduced in the modeled interface, from a buried

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**FIGURE 6.** The binding of huIgG dimers/trimers to mnFcγRIIa and mnFcγRIIb. Biotinylated huIgG dimers/trimers were titrated on transfectants expressing (A) huFcγRIIa (●) or mnFcγRIIa-allele 2 (♦) and mutants thereof wherein PMN(131–133) was replaced with HLD(131–133) of huFcγRIIa (■) and then further modified by additional replacement of PY(159–160) with LF(150–160) of huFcγRIIa (▲) or N135 replaced with T135 of huFcγRIIa (○); (B) huFcγRIIa (●) or mnFcγRIIa-allele1 (■) and mutants thereof wherein PY(150–160) was replaced with LF(159–160) of huFcγRIIa (▲) or where N135 was replaced with T135 of huFcγRIIa (○) or binding to untransfected cells only (×); (C) huFcγRIIb1 (+) or mnFcγRIIb2 (○) or mnFcγRIIb1 (◇) and mutants thereof wherein HM(131–132) was replaced with RS(131–132) of huFcγRIIb (◇). The binding of IgG to each receptor or mutated receptor was tested on at least three occasions.

**FIGURE 7.** Homology-based model of mnFcγRIIa complex with human IgG1-Fc. (A) Comparison of the FG loop and Trp residues of the “Trp sandwich” of mnFcγRIIa (cyan wire) or huFcγRIIa (magenta wire) interacting with human IgG1-Fc (yellow wire) or A chain (green wire). (B) Solvent-accessible surface view of huFcγRIIa complex with huIgG1-Fc and (C) solvent-accessible surface view of macaque FcγRIIa complex with huIgG1-Fc. Comparison of (B) and (C) indicates the critical Tyr^{157} of the FG loop has a reduced contact with huIgG1 in mnFcγRIIa. The Tyr^{157} residues of huFcγRIIa and mnFcγRIIa are shown in magenta and cyan, respectively. The IgG1-Fc chain A is shown in green, chain B in yellow, and FcγRIIa molecules are in light gray. Models were prepared based on the cocrystal structure of huFcγRIIa/huIgG1-Fc (Brookhaven Protein Data Bank ID 3RY6) (40).
surface area of 100 Å² to 69 Å², including the loss of a potential hydrogen bond (Fig. 7B, 7C). The interface contact is further diminished at Trp87 of mnFcγRIIa (68 Å² buried surface) compared with the complexed huFcγRIIa (82 Å² buried surface), which together with Trp110 form the so-called “Trp-sandwich” that binds Pro159 of the Fc A chain (Fig. 7A). These alterations to contact residues (Tyr157 and Trp87) can be attributed to the influence of the interspecies substituted residues Pro159 and Tyr160 in the G strand of mnFcγRIIa. The model predicts that the mnFcγRIIa Pro159 kinks the G strand and repositions Tyr157, reducing its conformational functional differences of high/low responder polymorphism of peripheral blood neutrophils from humans and Macaca nemestrina. Mononuclear cells were stained with anti-FcγRIIa mAb 8.7 and populations were identified by costaining with anti-CD20 (B cells), anti-CD14 (monocytes), anti-CD159a (NKG2) (M. nemestrina NK cells), or anti-CD56 (human NK cells). Plots are representative of three individual experiments involving four different animals or human volunteers.

Importantly, previous studies of huFcγRIIa support our binding and modeling data and are consistent with a role for Tyr160 in the macaque receptor in modulating interactions with IgG. Varying the sequence at position 160 of the human receptor by replacement of Phe160 with alanine substantially increased binding of huIgG1 and huIgG2. This is in agreement with cell distribution in rhesus and cynomolgus macaques (26, 27, 45), and increased expression of FcγRII on CD20+ B cells, CD14+ monocytes (Fig. 8), and platelets (not shown) and, as expected was absent from macaque CD159a* NK cells and human CD56* NK cells, which is in agreement with cell distribution in rhesus and cynomolgous macaques (26, 27, 45) and humans (reviewed in Refs. 1, 2). Considerable differences in FcγR expression were observed on neutrophils wherein FcγRII and FcγRIII were both expressed on human neutrophils (Fig. 9) but only FcγRII was present on M. nemestrina neutrophils and also at a level 5-fold greater than that detected on human cells. The absence of FcγRIII from macaque neutrophils has also been reported for rhesus and cynomolgus macaques (26, 45), and increased expression of FcγRII has also been shown in cynomolgus macaques (27).

Discussion

In this study, we demonstrate that human and M. nemestrina FcγRIIa and FcγRIIb have distinct hierarchies of binding of hu-IgG1 and IgG2. Although the FcγRIIa of both species binds IgG3 essentially equivalently, IgG1 and IgG2 binding to mnFcγRIIa is impaired by comparison with its human ortholog. Remarkably, the converse is the case for FcγRIIb where it is the human receptor that exhibits impaired binding of IgG1 relative to mnFcγRIIb, and IgG2 is not detectably bound. Indeed, mnFcγRIIb avidly binds IgG2 and thereby has a broader specificity for huIgG subclasses than does huFcγRIIb. These interspecies differences of IgG binding and specificity to mnFcγR that we describe in this study were determined in the physiological context of the cell surface. Similar results were observed in cell-free surface plasmon resonance analysis of recombinant ectodomains of cynomolgus FcγR, which showed lower affinity of huIgG1 and huIgG2 for cynomolgus FcγRIIa than huFcγRIIa, and conversely showed increased affinity of human IgG1 and IgG2.
for IgG1 and a greatly increased affinity of IgG2 for cynomolgus FcγRIIb compared with its human ortholog (27).

To identify key residues that contribute substantially to the observed interspecies IgG binding differences, we exchanged equivalent residues between the human and macaque FcR receptors in site-directed mutagenesis studies (Figs. 4–6). In short, Pro159 and Tyr160 contribute to the lower activity of the mnFcγRIIa, whereas His131 and Met132 are key to the higher activity of mnFcγRIIb compared with huFcγRIIb. Furthermore, we have also described the profound influence of polymorphism of mnFcγRIIa on IgG binding and identified FcγRIIa as highly polymorphic with eight alleles being detected in only 10 individuals.

The present studies have implications for the structural and functional analysis of IgG FcR interactions, not only in M. nemestrina but also in the widely used rhesus and cynomolgus macaques and other NHPs. A comparative analysis of FcγRIIa and FcγRIIb sequences of other NHPs shows that critical residues in the FG and C’E loops identified in this study as affecting the mnFcγRI interaction with huIgG are preserved in some but not all NHPs (Figs. 10–12).

The Pro159 and Tyr160 residues of the FG loop that adversely affect IgG binding by mnFcγRIIa are conserved in rhesus (M. mulatta) (Figs. 10, 11) and cynomolgus (M. fascicularis) macaque species (Fig. 11) (24, 27, 54), suggesting that these residues could be a key interspecies difference that modulates the engagement of huIgG in FcγRIIa of other macaque species such as cynomolgus FcγRIIa (27). Notably PY(159–160) are not conserved in other NHPs (Fig. 11), which raises the possibility that the diminished huIgG binding may be only a feature of macaque FcγRIIa.

In the case of position 131, which forms a major contact with huIgG, several, but importantly not all, NHPs also have histidine at this position in FcγRIIa, which could favor huIgG2 binding. His131 is also found in FcγRIIa of rhesus (M. mulatta) and cynomolgus (M. fascicularis) macaques, chimpanzee (Pan troglodytes), bonobo (Pan paniscus), baboon (Papio anubis), and squirrel monkey (Saimiri boliviensis), and thus it is likely that they also bind huIgG2. Indeed, rhesus and cynomolgus macaque FcγRIIa do bind huIgG2 as measured by surface plasmon resonance or immune complex binding to cells (27). However, orangutan (Pongo abelii) and marmoset (Callithrix jacchus) have Tyr131 and Arg131, respectively, and therefore may have altered huIgG binding compared with macaque FcγRIIa, especially with respect to huIgG2.

Our data also describe the profound influence of polymorphism in mnFcγRIIa on IgG binding, which adds additional complexity to analysis of huIgG interaction with macaque FcR. In M. nemestrina and interestingly in rhesus macaque, FcγRIIa is the most polymorphic of the FcγRs (24, 54) (Figs. 1, 2) However, only M. nemestrina showed sequence variation at position 131 wherein proline in this position in allele 2, also present in allele 5, results in ablation of IgG1 and IgG2 binding. This is the equivalent position to the clinically relevant high and low responder polymorphism in huFcγRIIa (11, 23) wherein allotypic receptor with Arg131 is hypofunctional with respect to IgG2 binding.

While the functional importance of FcR polymorphism on IgG binding in other macaque species has not been investigated, it is noteworthy that in rhesus macaques, polymorphism in the C’E loop at position 128 results in the presence/absence of a possible N-glycosylation site that replaces the Lys128 adjacent to Phe129, which in huFcγRIIa is a critical IgG contact (Fig. 10) (40). Interestingly, the same site is found in baboon and a unique site of possible N-glycosylation is present at position 133 in marmoset (Fig. 11), but whether these positions are polymorphic or functionally important in these species is unknown.

Thus, polymorphism, at least in FcγRIIa, is extensive (eight alleles in 10 animals), and it is likely that more alleles exist in M. nemestrina. Importantly, the presence of null or hypofunctional allotypic receptors is sufficiently frequent in M. nemestrina (two of eight allotypic receptors among 10 individuals) and potentially other species to warrant caution when interpreting results of studies involving models of Ab-based effects.

The inhibitory FcγRIIb also contains His131 in all major macaque species widely used in research, including pig-tailed (M. nemestrina), rhesus (M. mulatta), and cynomolgus (M. fascicularis) macaques as well as the baboon (P. anubis) (Fig. 12). Based on the M. nemestrina data in this study, the FcγRIIB of these species is likely to bind human IgG1, and importantly IgG2, more avidly than huFcγRIIb. Indeed, avid binding of IgG2 has been observed in cynomolgus FcγRIIb (27). In all other NHP species, arginine is preserved at this position, which suggests FcγRIIB of these species may behave more similar to huFcγRIIb with reduced immune complex binding and little IgG2 binding, making the FcγRIIB of macaques unique in this regard.

In humans, subtle affinity differences have indeed been shown to be critically important in the respective functions of human FcγRIa and FcγRIIB (53). Because of the altered specificity of mnFcγRIIb and/or the reversed hierarchy of IgG1 and IgG2 binding to mnFcγRIIa and mnFcγRIIb, it is conceivable that Ab therapeutics, especially IgG2, may not behave in M. nemestrina or other macaque species as they may be expected to in humans. The
Increased binding to mmFcRRIIb may obscure potent and desirable effector function or alternatively obscure adverse reactions that may otherwise be manifested in humans where affinity for the inhibitory FcRIIb is lower. Furthermore, with the success of mAb therapy (1), efforts have been made to alter the potency of useful therapeutic mAbs that include specific engineered changes to the IgG-Fc region to optimize the interaction with huFcRs (21, 22, 55). However, the FcRIIa or FcRIIb selectivity of such engineered Abs may not necessarily exhibit improved binding to macaque FcRs (56).

Thus, our data highlight that the activities of mAbs designed to alter interactions between human Abs and huFcRs may not be faithfully recapitulated in preclinical studies in nonhuman primates, or at least in macaques.

Similar caveats may apply to viral pathogenesis studies in macaques of human infections where FcRIIa or FcRIIb are involved in significant clinical or biological aspects of the natural history of the infection in humans, for example, Ab-dependent enhancement (57) in dengue infection or skewing of the FcRIIa/FcRIIb expression ratio in HIV infection (12) or resistance to HIV (13, 14).

Thus, the interspecies and polymorphic differences we describe in this study may translate to alterations of Ab-induced inflammatory outcomes in vivo in NHPs that are distinct from those in humans.

When considering the impact of differences in FcR binding function in vivo in NHPs, it is important to consider whether any differences in cell distribution may also confound the interpretation of in vivo studies of Ab function. The cell distribution of FcR is for the most part similar to humans in M. nemestrina (Figs. 8, 9) and in rhesus (26, 45) and cynomolgus macaques (27), with the notable exception of neutrophils where FcRIII is lacking but FcRII is elevated. Thus, the implication is that differences in cell distribution in M. nemestrina or other macaque species may translate to alterations of Ab-induced inflammatory outcomes in vivo in NHPs that are distinct from those in humans.
not affect the evaluation of mAbs except where effector function is dependent on the FcRys of neutrophils.

Although the macaque is a valuable model of human immune function, clear differences exist between species. Cautious interpretation of responses involving Ab-induced responses is prudent. Clearly analysis of NHP FcγR genotype and binding profiles of therapeutic IgG to receptors may be useful in the design of experimental or preclinical studies. Thus, the use of NHPs as a model of human immunity or preclinical model for the evaluation of Ab FcR interactions will be greatly assisted by identifying and understanding the basis for the differences in interaction of macaque and huFcR with huIgG.

Disclosures

The authors have no financial conflicts of interest.

References


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