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 Macaca nemestrina activating FcγRIIa (mnFcγRIIa) and inhibitory FcγRIIb (mnFcγRIIb) and predicted their structures using the huIgG/Fc/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 (H131). In contrast, mnFcγRIIb has enhanced binding of huIgG1 and broader specificity, as, unlike huFcγRIIb, it avidly binds IgG2. 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 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 consequently Ab responses. FcγRIib also regulates signaling by the activating FcRs FcγRI, FcγRIIa, FcγRIIb, FcγRI, and FcγRI. 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 FcyRIIa (21) or inhibitory FcRIIB (22). Furthermore, the most clinically significant polymorphism of the activating IgG receptor, FcyRIIa (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 complicate interpretation owing to nonsynonymous polymorphisms 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 FcγRIIa 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 FcyRs**

Gene transcripts for mnFcγRIla 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 FcyR 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 FcyRIIa or FcyRIIb were based on sequences of rhesus macaque (*M. mulatta*, GenBank NM_001257300) or cynomolgus macaque (*M. fascicularis*, GenBank AF485814.1), respectively. The FcyRIIa 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 FcyRIIb 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 KpnI/EcoRV digested PCR products were cloned into PENTRI1 (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 FcyRIIb (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 FcyRII sequences were generated using CLC Sequence Viewer version 6.4 (CLC bio, Aarhus, Denmark).

Isolation and expression of the human FcyRIIa-H131 and FcyRIIa-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, P159L,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 mnFcγRIIb1 (mutant 6, H131R, M132S [HM→RS]).

**Stable expression of FcyRII**

A pMXI retroviral expression system was used to introduce FcyRII 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 FcyRIII 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 IgG1a, 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 ImmunoResearch Laboratories (West Grove, PA). Human IgG in the form of i.v. Ig Sandoglobulin was obtained from Novartis (Sydney, NSW, Australia).

Abs used to identify populations of peripheral blood leukocytes were anti-CD20, (clone L27; BD Biosciences, Sydney, NSW, Australia), anti-CD14 (clone n52; BD Biosciences), anti-CD56 (clone NCA162; BD Biosciences), anti-CD159a (NKG2) (clone Z199; Beckman Coulter, Melbourne, VIC, Australia), anti-FcyRIII (clone 3G8; BD Biosciences), and anti-CD41a (clone HIP8; BD Biosciences).
The anti-FcγRIIA mAbs 8.7, 8.2, and IV3 and the anti-FcγRIIB monoclonal 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 IgF(β)2 at a 1:1 ratio for 30 min at 37°C and then on ice for 5 min. Second, for IgG dimers/trimers, biotinylated IgG dimers/trimer complexes of pooled huIgG were generated using the cross-linker Tris-succinimidyldiaminotriacetate (Thermo Scientific). Tris-succinimidylaminotriacetate 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-succinimidyldiaminotriacetate, which was used without purification. Human IgG (4 ml at 25 mg/ml in PBS) was mixed with an 11-fold excess of biotinyl bis-succinimidyldiaminotriacetate (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 fluorochrome-conjugated goat anti-human IgG 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 FcγRIIA and FcγRIIB were determined in each experiment by flow cytometry using the following biotinylated anti-FcγRIIA mAb F(ab′)2 fragments: 8.7, 8.2, and Fab(β)2 of 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 from 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 monocytes were identified with anti-CD14. Human NK cells were identified with anti-CD56 and macaque NK cells with anti-CD159a (NKG2) (42). 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 FcyRIIa was defined using clone 3G8, and FcyRIII 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 huFcyRIIA/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 mFcγRIIA-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 huFcyRIIA and mnFcγRIIA-allele 1. Because mFcγRIIA-allele 1 has one additional N-linked glycosylation site compared with huFcyRIIA, 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 huFcyRIIA 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 huFcyRIIA 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. 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 huFcyRIIA, with seven or eight of the nine polymorphic positions, respectively, being distinct from huFcyRIIA (Fig. 1).

In M. nemestrina, the inhibitory FcγRIIB has 23 nonpolymorphic amino acid differences from huFcyRIIB, and 21 of these occur in the extracellular domains. A single polymorphism resulting in amino acid substitution methionine/valine at position 254 was apparent in the cytoplasmic tail (Fig. 2).

Binding of huIgG to mnFcγRIIA

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

The binding of huIgG3 complexes to mnFcγRIIA-allele 1 and huFcyRIIA-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 huFcγRIIa-His131 (Fig. 3B, 3C). Thus, in comparison with huFcγRIIa, the mnFcγRIIa-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 FcγRIIa observed above, even greater differences were found in huIgG binding to the allelic forms of mnFcγRIIa (Fig. 4). The binding of all huIgG subclasses to mnFcγRIIa-allele 2 receptor was reduced greatly (Fig. 4B–D). Compared to binding by mnFcγRIIa-allele 1 (MFI of 5000), IgG1 binding to mnFcγRIIa-allele 2 was reduced 80-fold (MFI of 90) and was barely detectable above background (MFI of 30, Fig. 4B). Similarly, IgG2 binding of mnFcγRIIa-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 mnFcγRIIa-allele 1 (MFI of 70,300). Thus, not only do interspecies differences of FcR affect huIgG binding, but polymorphisms (intraspecies differences) in mnFcγRIIa also influence the binding of huIgG. The mnFcγRIIa-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 mnFcγRIIa-allele 2. Analysis of the polymorphic amino acid differences in the mnFcγRIIa in the context of known functional regions of huFcγRIIa showed that position 131

FIGURE 1. Alignment of the eight allelic forms of FcγRIIa of M. nemestrina with their human counterpart (huFcγRIIa-His131, UniProt P12318.4) (41). Amino acids identical to M. nemestrina allele 1-encoded receptor are shown as dots, and positions of polymorphism are indicated by inverted triangles; the transmembrane region is shown as a dashed underline. Critical residues analyzed in this study are boxed. Trp87 and Trp110 are indicated with stars. These sequences were derived from 10 animals. The M. nemestrina sequences are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank) as follows: mnFcγRIIa-allele 1, accession no. KF234399; mnFcγRIIa-allele 2, accession no. KF234400; mnFcγRIIa-allele 3, accession no. KF234401; mnFcγRIIa-allele 4, accession no. KF234402; mnFcγRIIa-allele 5, accession no. KF562260; mnFcγRIIa-allele 6, accession no. KF562261; mnFcγRIIa-allele 7, accession no. KF562262; and mnFcγRIIa-allele 8, accession no. KF562263.
is part of the major contact surface in the huFcγRIIa/IgG inter-
action (40, 49, 50). Moreover, in humans, polymorphic FcγRIIa
(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 mnFcγRIIa-allele 1
compared with the PMN(131–133) of the poorly functional receptor
encoded by mnFcγRIIa-allele 2 (Fig. 1).

Consequently, we attempted to reconstitute binding of huIgG by
replacing PMN(131–133) of mnFcγRIIa-allele 2 with HLD(131–133)
from huFcγRIIa. Note that His131 and Asp133 of mnFcγRIIa-
allele 1 are identical in huFcγRIIa (Fig. 1). Weak binding of IgG1
and IgG2 to mnFcγRIIa-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, re-
spectively (Fig. 4F, 4G; blue filled, dashed line) that was com-
parable to that of mnFcγRIIa-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 huFcγRIIa-His131; that is, MFIs of 57,200 and
3,600, respectively. Binding of IgG3 by the mnFcγRIIa-allele 2
with HLD(131–133) remained slightly reduced compared with the
human receptor (MFI of 53,400 for mnFcγRIIa-His131 compared
with 70,100 for huFcγRIIa-His131; compare with Fig. 3). The
failure to fully enable IgG binding to levels observed for the hu-
man receptor implied that other amino acid residue differences
between mnFcγRIIa and huFcγRIIa contribute to ligand binding.

Of particular interest in this context were residues Pro159 and
Tyr160 in mnFcγRIIa and Leu159 and Phe160 in huFcγRIIa, which are
located in the G strand of the second domain adjacent to the critical
FG loop that contacts IgG.

Thus, allele 2–encoded mnFcγRIIa, which we had mutated to
contain the HLD(131–133) of huFcγRIIa-His131 (Fig. 4E–H,
dashed line), was further modified by replacing Pro159 and Tyr160
in mnFcγRIIa and Leu159 and Phe160 in huFcγRIIa, which are
located in the G strand of the second domain adjacent to the critical
FG loop that contacts IgG.

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

FIGURE 2. Alignment of the allotypic form of FcγRIIb of M. nemestrina with the splice variants of its human counterpart (huFcγRIIb1, GenBank NM_001002275.2; huFcγRIIb2, GenBank NM_001002273.2) (38). Amino acids identical to M. nemestrina are shown as dots, and positions of poly-
morphism are indicated by inverted triangles; the transmembrane region is shown as underlined dashed line, and the deletions within the cytoplasmic tail of FcγRIIb2 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: mnFcγRIIb1, accession no. KF234403; and mnFcγRIIb2, accession no. KF234404.

FIGURE 3. Comparative binding of huIgG subclasses to mnFcγRIIa-allele 1 (red filled histogram with dashed red line) or the huFcγRIIa-His131 (open histogram, solid red line). Cell surface expression of receptor protein was determined using the anti-FcγRII mAb 8.7, which recognizes huFcγRIIa and mnFcγRIIa. 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 bind IgG2 was surprising because in humans IgG2 essentially

mnFc

similar levels (Fig. 5D).

principal sequence differences occur around position 131 wherein (159–160) was replaced with human LF(159–160)

as in (Fig. 5). Indeed, unlike the activating FcγRIIa-allele 1 (red filled histogram, dashed line) and background binding (black line). Binding of anti-receptor mAb 8.7 (I) or IgG (J–L) to

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 mnFcγRIIa results in a significant impairment of binding of the human IgG1 and IgG2 subclasses.

Binding of huIgG to mnFcγRIIb

The interaction of huIgG subclass 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 complex, 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 Tyr157, conserved in all macaque species and humans, makes critical contacts with Leu234 and Gly236 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 Tyr157 of mnFcγRIIa FG loop and the IgG-Fc are reduced in the modeled interface, from a buried
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 Trp⁷⁰ 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 Pro¹⁵⁹⁰ 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 mmFcγRIIa. The model predicts that the mmFcγRIIa Pro¹⁵⁹ kinks the G strand and repositions Tyr¹⁵⁷, reducing its conformational differences of high/low responder polymorphism of the macaque receptor in modulating interactions with IgG. Varying Phe¹⁶⁰ with alanine substantially increased binding of the sequence at position 160 of the human receptor by replacing the macaque receptor with Tyr160 side-chain packs against Trp⁸⁷, reducing contact in the Trp sandwich of Trp⁶⁷ with Pro¹³⁹ of the IgG-Fc A chain (Fig. 7A). The structural effect of these residues PY(159–160) on the critical contact residues agrees with the mutagenesis data (Figs. 4, 6), which showed that the replacement of the PY(159–160) of mmFcγRIIa with LF(159–160) of huFcγRIIa optimized the interaction with huIgG1 and huIgG2.

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 both huIgG1 and huIgG2 (50, 52). Conversely, the replacement of Phe¹⁶⁰ with tyrosine reduced binding of IgG (53); note that tyrosine occupies this position in mmFcγRIIa (Fig. 1). Thus, it is likely that the improved binding of huIgG by the mutated mmFcγRIIa following our replacement of Pro¹⁵⁹–Tyr¹⁶⁰ with Leu¹⁵⁹–Phe¹⁶⁰ of huFcγRIIa results from a more favorable orientation of the contact Tyr¹⁵⁷ and of the Trp⁷⁰/Trp¹¹⁰ sandwich in the interaction with human IgG-Fc (Fig. 7).

Another major contact of FcγRIIa in complex with Fc is made by the C’E loop, especially residue 131, which determines the functional differences of high/low responder polymorphism of FcγRIIa in humans. Arg¹³¹ is accommodated in a shallow depression in the Fc and is somewhat “crowded,” but the His¹³¹ is more readily accommodated (40), and in the mmFcγRIIa-allele 1 and mmFcγRIIb, which also contain His¹³¹, this also is likely to be the case. In the case of mmFcγRIIα-allele 2, the nearly complete loss of binding caused by the presence of proline in position 131 is likely due to a major alteration of the C’E loop structure leading to the disruption of the interface with IgG.

Expression of FcγRII in M. nemestrina blood cells

The distribution of FcγRII on leukocyte types has been determined for rhesus and cynomolgous macaques (26, 27, 45), and we analyzed expression in M. nemestrina. Flow cytometry analysis of peripheral blood leukocytes from four macaques confirmed the 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 CD56bright 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 FcyRII and FcyRIII were both expressed on human neutrophils (Fig. 9) but only FcyRII was present on M. nemestrina neutrophils and also at a level 5-fold greater than that detected on human cells. The absence of FcyRIII from macaque neutrophils has also been reported for rhesus and cynomolgous macaques (26, 45), and increased expression of FcyRII 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 FcyRIIa of both species binds IgG3 essentially equivalently, IgG1 and IgG2 binding to FcyRIIa is impaired by comparison with its human ortholog. Remarkably, the converse is the case for FcyRIIB where it is the human receptor that exhibits impaired binding of IgG1 relative to FcyRIIB, and IgG2 is not detectably bound. Indeed, mmFcγRIIB avidly binds IgG2 and thereby has a broader specificity for huIgG subclasses than does huFcγRIIB. These interspecies differences of IgG binding and specificity to FcγRI-R 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 FcyRIIa than huFcγRIIa, and conversely showed increased affinity for M. nemestrina FcγRIIIa.
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 mmFcγRⅡb compared with huFcγRⅡb. Furthermore, we have also described the profound influence of polymorphism of mmFcγRⅡa 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 mmFcγRⅡ 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 mmFcγRⅡa 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 huIgG 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 huIgG. Indeed, rhesus and cynomolgus macaque FcγRⅡa do bind huIgG 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 mmFcγRⅡa 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 FcyRs (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 Lys129 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 FcγRⅡa and FcγRⅡb (53). Because of the altered specificity of mmFcγRⅡb and/or the reversed hierarchy of IgG1 and IgG2 binding to mmFcγRⅡa and mmFcγRⅡb, 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.

**FIGURE 10.** Structural location of human and macaque interspecies sequence diversity and macaque polymorphism of FcγRⅡa. The locations of polymorphic or nonpolymorphic amino acids are shown on the α carbon trace of residues 4–170 of huFcγRⅡa. Cyan spheres show the location of nonpolymorphic amino acids that are identical to both M. nemestrina (A) and M. mulatta (B) but differ from huFcγRⅡa. The red spheres indicate the location of residues where polymorphism is unique to one macaque species. The yellow spheres indicate location of residues where polymorphism has been identified in both M. nemestrina and M. mulatta. Sequence numbering follows that of Fig. 1.
increased binding to mmFcRIIb may obscure potent and desirable effector function or alternatively obscure adverse reactions that may otherwise be manifested in humans where affinity for the inhibitory FcγRIIb 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 huFcγRs (21, 22, 55). However, the FcγRIIa or FcγRIIb selectivity of such engineered Abs may not necessarily exhibit improved binding to macaque FcγRs (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 FcγRIIa or FcγRIIb 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 FcγRIIa/FcγRIIb expression ratio in HIV infection (12) or resistance to HIV (13, 14).

When considering the impact of differences in FcγR 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 FcγR 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 FcγRIII is lacking but FcγRII is elevated. Thus, the implication is that differences in cell distribution in M. nemestrina or other macaques species may

**FIGURE 11.** Alignment of the extracellular domains of NHPs, including rhesus allelic forms and huFcγRIIa. For additional allelic forms of *M. nemestrina*, see Fig. 1. Amino acids identities are shown as dots. Critical residues analyzed in this study are boxed. Species shown are pig-tailed macaque (*M. nemestrina*), rhesus macaque (*M. mulatta*) (24), cynomologus macaque (*Macaca fiscularis*, GenBank AF485813) (27), baboon (*P. anubis*, GenBank XM_003892972.1), chimpanzee (*P. troglodytes*, UniProt Q8SPV8.1), bonobo (*P. paniscus*, GenBank XM_003804397.1), orangutan (*P. abelii*, GenBank XM_002809886.2), squirrel monkey (*S. boliviensis*, GenBank XM_003943372.1), and marmoset (*C. jacchus*, GenBank XM_003735165.1). The known allelic forms with polymorphisms in the extracellular region are shown for *M. nemestrina* and *M. mulatta*.

**FIGURE 12.** Alignment of the extracellular domains of NHPs and huFcγRIIb. Amino acids identities are shown as dots. Critical residues analyzed in this study are boxed. Shown are pig-tailed macaque (*M. nemestrina*), rhesus macaque (*M. mulatta*) (24), cynomologus macaque (*M. fiscularis*) (27), baboon (*P. anubis*, GenBank XM_003892974.1), chimpanzee (*P. troglodytes*, GenBank XM_001153863.2), bonobo (*P. paniscus*, GenBank XM_003824761.1), orangutan (*P. abelii*, GenBank XM_002809886.2), squirrel monkey (*S. boliviensis*, GenBank XM_003943370.1), and gorilla (*Gorilla gorilla*, GenBank XM_004027772).
not affect the evaluation of mAbs except where effector function is dependent on the FcRs 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 Fc effector function, clear differences exist between species. Cautious interpretation of responses involving Ab-Fc interactions will be greatly assisted by identifying and understanding the basis for the differences in interaction of macaque and huFcR with hulG.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


