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Different Adaptations of IgG Effector Function in Human and Nonhuman Primates and Implications for Therapeutic Antibody Treatment

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Safety of human therapeutic Abs is generally assessed in nonhuman primates. Whereas IgG1 shows identical FcγR interaction and effector function profile in both species, fundamental differences in the IgG2 and IgG4 Ab subclasses were found between the two species. Granulocytes, the main effector cells against IgG2- and IgG4-opsonized bacteria and parasites, do not express FcγRIIIb, but show higher levels of FcγRII in cynomolgus monkey. In humans, IgG2 and IgG4 adapted a silent Fc region with weak binding to FcγR and effector functions, whereas, in contrast, cynomolgus monkey IgG2 and IgG4 display strong effector function as well as differences in IgG4 Fab arm exchange. To balance this shift toward activation, the cynomolgus inhibitory FcγRIIB shows strongly increased affinity for IgG2. In view of these findings, in vitro and in vivo results for human IgG2 and IgG4 obtained in the cynomolgus monkey have to be cautiously interpreted, whereas effector function-related effects of human IgG1 Abs are expected to be predictable for humans. The Journal of Immunology, 2012, 188: 000–000.
and toward IgG4, which (together with IgE) is generated in antigen-driven immunity and responses to allergens. In contrast, IgG1 and IgG3 subclasses, which are mainly involved in other types of the humoral immune response, show a very similar pattern of FcγR binding and effector function in the two species. To our knowledge, this study describes for the first time the complete set of FcγR/IgG interactions and functional consequences in the cynomolgus monkey in comparison with the human system, and will therefore be very helpful in interpreting the data generated with therapeutic Abs in NHP and their translation to humans.

Materials and Methods

Recombinant FcγR and Abs

Extracellular domains of human and cynomolgus FcγR were cloned into expression vectors harboring a 4-aa purification tag (4APP; Novartis) and an Avi biotinylation tag (GLNDIFEAQIKIEWHE; Avidity). Puriﬁed Ab clones 27–35 [mouse IgG2b, 22], 3D3-FITC, and FLI8.26-FITC were site directed biotinylated with BirA (Avidity). Extracellular domain of human FcγRI (UniProt: P01857, 1-16) instead of the natural leader and the CD33 leader showed identical amino acid sequences as analyzed by mass spectrometry. The proteins expressed with the natural leader and the CD33 leader showed a 3-fold higher expression of FcγRI (CD64) protein was purchased from R&D Systems (Minneapolis, MN). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929) were cloned from cynomolgus blood mRNA (primer: κ L chain, 5’-ATCAAACGAGCTGTGGCTGCA-CA-3’, 5’-ATTTACACCTCTCCCTTGA-3’, γ H chain, 5’-GGCTC-CAACAGGGCCCATCG-3’, 5’-TTTACCCGGAGACACCGAGAG-3’). Genomic DNA from the blood of 10 cynomolgus animals was sequenced (Detecto, Minneapolis, MN). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929) were cloned from cynomolgus blood mRNA (primer: κ L chain, 5’-ATCAAACGAGCTGTGGCTGCA-CA-3’, 5’-ATTTACACCTCTCCCTTGA-3’) and γ H chain C regions (primer: γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929) were cloned from cynomolgus blood mRNA (primer: κ L chain, 5’-ATCAAACGAGCTGTGGCTGCA-CA-3’, 5’-ATTTACACCTCTCCCTTGA-3’, γ H chain, 5’-GGCTC-CAACAGGGCCCATCG-3’, 5’-TTTACCCGGAGACACCGAGAG-3’). Genomic DNA from the blood of 10 cynomolgus animals was sequenced (Detecto, Minneapolis, MN). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929) were cloned from cynomolgus blood mRNA (primer: κ L chain, 5’-ATCAAACGAGCTGTGGCTGCA-CA-3’, 5’-ATTTACACCTCTCCCTTGA-3’, γ H chain, 5’-GGCTC-CAACAGGGCCCATCG-3’, 5’-TTTACCCGGAGACACCGAGAG-3’). Genomic DNA from the blood of 10 cynomolgus animals was sequenced (Detecto, Minneapolis, MN). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929) were cloned from cynomolgus blood mRNA (primer: κ L chain, 5’-ATCAAACGAGCTGTGGCTGCA-CA-3’, 5’-ATTTACACCTCTCCCTTGA-3’, γ H chain, 5’-GGCTC-CAACAGGGCCCATCG-3’, 5’-TTTACCCGGAGACACCGAGAG-3’). Genomic DNA from the blood of 10 cynomolgus animals was sequenced (Detecto, Minneapolis, MN). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984926; IgG4, GenBank: JN984929) were cloned from cynomolgus blood mRNA (primer: κ L chain, 5’-ATCAAACGAGCTGTGGCTGCA-CA-3’, 5’-ATTTACACCTCTCCCTTGA-3’, γ H chain, 5’-GGCTC-CAACAGGGCCCATCG-3’, 5’-TTTACCCGGAGACACCGAGAG-3’).

Results

Different FcγRI expression on human and cynomolgus granulocytes

FcγR fulfill diverse functions in the different immune cells from neutrophil Ab-mediated phagocytosis of bacteria to regulation of B cell activation.

Human granulocytes express only FcγRIIb and FcγRIIa, and therefore become highly activated by IgG-opsonized bacteria or parasites. In contrast to humans, NHP do not express any form of FcγRIII on granulocytes (21). Surprisingly, we found instead a 3-fold higher expression of FcγRII on cynomolgus granulocytes in comparison with humans (Fig. 1). This is a speciﬁc diﬀerence in the FcγR expression pattern of neutrophils as other populations such as monocytes (Fig. 1), B cells, or NK cells (data not shown) show no diﬀerences in the expression pattern and levels between human and cynomolgus monkey. As FcγRIII has a diﬀerent binding proﬁle in comparison with FcγRII, especially for the IgG2 subclass (22), we decided to perform a complementary analysis of the whole IgG/FcγR interaction set in humans and the cynomolgus monkey.

Cynomolgus IgG2 and IgG4 show increased binding to FcγRII and FcγRI

Highly puriﬁed monomeric Abs with VH and VL derived from the CD20-binding Ab rituximab (19) recombinantly expressed with the four human and cynomolgus IgG subclasses were used to


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measure their binding against the different human and cynomolgus FcγRs by surface plasmon resonance. In contrast to human IgG2 and IgG4 isotypes, which show weak interaction with the human and cynomolgus activating receptors FcγRI and FcγRIII, cynomolgus IgG2 and IgG4 showed substantial binding to corresponding FcγRII of both species (Fig. 2). Human and cynomolgus FcγRII do not bind to human IgG2, but show nanomolar affinity to cynomolgus IgG2 (Fig. 2A, 2B, Supplemental Figs. 2, 3, Table I). The binding of FcγRI to cynomolgus IgG4 shows a 10× higher affinity due to a slower off-rate in comparison with human IgG4 (Fig. 2B). Human and cynomolgus FcγRIII bind cynomolgus IgG2 and IgG4 with a 5–10× higher affinity than human IgG2 and IgG4 (Fig. 2D–G). Overall, human and cynomolgus FcγRI and FcγRIII show a qualitatively similar binding profile toward the different human and cynomolgus IgG isotypes (IgG1–IgG4). In humans, FcγRIII has an allotypic variation and FcγRIII-158V has a higher affinity than FcγRIII-158F (23). Interestingly, cynomolgus FcγRIII contains the more valine-like amino acid isoleucine at position 158 and accordingly shows a high-affinity phenotype (Table I). Distinct amino acid substitutions in the cynomolgus IgG sequences at the Fc binding region of cynomolgus FcγRIIa (131H) show a higher affinity to IgG2 than human FcγRIIa-131H and cynomolgus FcγRIIb, in which the amino acid at position 131 is an arginine leading to low-affinity binding of FcγRIIb, cynomolgus FcγRIIa has a high-affinity conferring histidine at this position (Fig. 3C). Accordingly, cynomolgus FcγRIIB shows a 20-fold higher affinity to human IgG2 and a 10-fold higher affinity to cynomolgus IgG2 compared with human FcγRIIB (Fig. 2C).

IgG4 Fab arm exchange

Another important feature that distinguishes cynomolgus IgG4 from human IgG4 is its inability to perform Fab arm exchange. It is now well established that human IgG4 can efficiently perform

![FIGURE 1. FcγRII expression on human and cynomolgus granulocytes (A) and monocytes (B) distinguished by forward and sideward scatter intensity. Human donors were simultaneously analyzed for the different FcγRII allotypes (FcγRIIa 131 HH, HR, RR). Cynomolgus granulocytes expressed significantly higher levels of FcγRII in comparison with all human individuals and to each subgroup of the FcγRIIa allotypes (**p < 0.0001). No difference was found in FcγRII expression between human and cynomolgus monocytes.

![FIGURE 2. Binding of human and cynomolgus IgG isotypes to Fcγ-R. (A–G) Binding of the different isotypes to (A) human FcγRI, equilibrium-binding constants (K_a); (B) human FcγRII, dissociation rates (k_off); (C) FcγRIIIa, K_a, and FcγRIIIb, K_a; (D–G) binding levels at equilibrium: (D, E) human FcγRIIa, (F, G) cynomolgus FcγRIIIa. Human IgG1 (black circle), human IgG2 (green circle), human IgG3 (blue circle), human IgG4 (red circle), cyno IgG1 (black triangle), cyno IgG2 (green triangle), cyno IgG3 (blue triangle), cyno IgG4 (red triangle). n = 2 for all experiments; (A–C) mean values of two independent experiments; (D–G) values in duplicates; one representative experiment of two is shown.]
Table I. Overview of binding affinities to FcγR and ADCC potency of the different anti-CD20–based human and cynomolgus IgG isotypes

<table>
<thead>
<tr>
<th></th>
<th>Human IgG1</th>
<th>Human IgG2</th>
<th>Human IgG3</th>
<th>Human IgG4</th>
<th>Cyno IgG1</th>
<th>Cyno IgG2</th>
<th>Cyno IgG3</th>
<th>Cyno IgG4</th>
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<tr>
<td><strong>Cellular binding</strong> (n = 2)</td>
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<tr>
<td>Cyto FcγRI EC₅₀ (nM)</td>
<td>0.20 ± 0.09</td>
<td>&gt;30⁰</td>
<td>0.13 ± 0.05</td>
<td>0.29 ± 0.04</td>
<td>0.14 ± 0.03</td>
<td>0.40 ± 0.11</td>
<td>0.31 ± 0.07</td>
<td>0.30 ± 0.18</td>
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<tr>
<td>Biacore binding (n = 2)</td>
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<tr>
<td>Human FcγRI K₂ₑ (nM)</td>
<td>0.17 ± 0.01</td>
<td>No binding</td>
<td>0.13 ± 0.03</td>
<td>1.24 ± 0.37</td>
<td>0.17 ± 0.02</td>
<td>0.48 ± 0.12</td>
<td>0.23 ± 0.04</td>
<td>0.35 ± 0.1</td>
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⁰Value is extrapolated and is outside the range of the used concentrations.

half-molecule Fab arm exchange in vivo, which is thought to further decrease the effector function of this isotype by preventing target cross-linking (27, 28). IgG4 Fab arm exchange is dependent on the presence of a serine at position 228 in the hinge of the IgG4 molecule. In contrast to human IgG4, however, we identified a cynomolgus IgG4 genotype with a proline-containing IgG1-like hinge sequence (Fig. 3A) in all 12 individual cynomolgus monkeys analyzed in this study that was resistant to Fab arm exchange (Fig. 4). We could not detect the generation of anti-CD20/anti-CD28–bisspecific Abs if cynomolgus IgG4 subclass Abs were combined with either human, cynomolgus, or rhesus IgG4 Abs. In contrast, human and rhesus IgG4 anti-CD20/anti-CD28 Ab combinations generated high levels of bispecific Abs in vitro as well as in a mouse in vivo system (Fig. 4A, 4B). The cynomolgus IgG4 H chain is missing a cysteine at position 131 in comparison with human IgG4, and thus, the H and L chains are only held together via noncovalent interactions (20, 28). However, introduction of the cysteine (S131C) did not result in enhancement of Fab arm exchange (Fig. 4B). Notably, cynomolgus IgG4 with a human IgG4-like hinge region (P238S) did not increase Fab arm exchange. In contrast, the C₃₃ domain of cynomolgus IgG4 seems to be important for Fab arm exchange, because the exchange of this domain by the human or rhesus C₃₃ completely restored the Fab arm exchange with human IgG4 (Fig. 4B). To demonstrate the generation of bispecific Abs by Fab arm exchange by an alternative method, we used LC-MS under denaturing conditions and the cysteine-containing version of cynomolgus IgG4 (S131C). Combinations of human/humanIgG Abs generated a third mass exactly between the masses of the two human parental Abs. In contrast, combination of human and cynomolgus IgG4 Abs did not reveal an additional mass species, further providing evidence that cynomolgus IgG4 does not perform half-molecule exchange (Fig. 4C–F).

Cynomolgus IgG2 and IgG4 show enhanced effector function

To analyze whether the observed differences in human and cynomolgus IgG2 and IgG4 at the molecular and biochemical level are reflected in different function of these subclasses, we used human and cynomolgus PBMCs and serum to assess the ADCC and CDC potency of anti-CD20 Abs grafted on human and cynomolgus C region IgG subclasses. Cynomolgus B cells include two different populations characterized by different relative expression of CD20, CD40, and CD21 (i.e., CD20<sup>low</sup>CD40<sup>hi</sup>CD21<sup>−</sup> and CD20<sup>high</sup>CD40<sup>low</sup>CD21<sup>hi</sup>) (29). Because the CD20<sup>low</sup>CD40<sup>hi</sup>CD21<sup>−</sup> population in cynomolgus monkey shows similar properties as human B cells with respect to in vivo and in vitro sensitivity to rituximab treatment, we analyzed the in vitro depletion of CD40<sup>high</sup>CD21<sup>−</sup> B cells in both species to obtain comparable data. Human IgG2 and IgG4 are functionally silent not only toward human, but also toward cynomolgus PBMC, because they show similar low binding to human and cynomolgus FcγRIII (Fig. 2D, 2F), resulting in a corresponding similar low ADCC activity with human and cynomolgus NK cells (Fig. 5A, 5C). In contrast to human IgG2 and IgG4, cynomolgus IgG2 and IgG4 show potent ADCC with both human and cynomolgus PBMC as well as potent CDC with human and cynomolgus serum (Fig. 5B, 5D, Supplemental Fig. 4). In contrast, IgG1 and IgG3, the subclasses with equivalent FcγR binding, show a similar effector function profile on both human and cynomolgus PBMCs (Fig. 5). In conclusion, the observed ADCC and CDC functional activities appear to directly correlate with critical amino acids and binding data differences of the human and cynomolgus IgG subclasses. In contrast to the human IgG2 and IgG4 subclasses, cynomolgus IgG2 and IgG4 are not silent, whereas IgG1 and IgG3 behave very similar in the two species.

Discussion

Despite the reported sequence homology of human and cynomolgus IgG subclasses and FcγRs (18), we could identify key differences between these two species on the level of molecular sequences, FcγR binding, receptor expression, distribution, and function. The identified differences induce changes, which can be explained by a single divergent evolutionary constraint, namely a different strategy to fight against bacteria and parasites. Cyno-
further increased by mutations in the hinge and CH3 abrogating humans. The potential effector function of cynomolgus IgG4 is show an almost identical profile in both cynomolgus monkeys and binding and effector function, whereas IgG1 and IgG3 subclasses and allergens preferentially induce the generation of IgG4 (and specific IgG2 subclass Abs (16, 17), whereas helminth parasites molecular sequence or allotype was not analyzed in this study (35).

Of note, we found that the increase of activating function of cynomolgus IgG2 (i.e., higher binding to FcγRI and FcγRIII and higher effector function) is counterbalanced by a strongly increased affinity toward cynomolgus FcγRIIb. Interestingly, in contrast to FcγRIIb in the NHP macaque family members cynomolgus and rhesus monkey, which contain a histidine at the position 131, all human primates analyzed (gorilla, orangutan, and chimpanzee) are characterized by an arginine at position 131. This change toward the low-affinity arginine variant of FcγRIIb is always accompanied by a corresponding low-afffinity mutation in the Fc binding region of IgG2 similar to the mutation found in human IgG2 (Fig. 3B, 3C). We therefore hypothesize that the inhibitory receptor FcγRIIb and the silent isotypes coevolved and that macaque species such as the cynomolgus and rhesus monkey have developed a different strategy to balance IgG/FcγR interactions in contrast to humans and human primates. This coevolution of activating IgG isotypes and the inhibitory FcγR, FcγRIIb, supports the model of activating-to-inhibitory ratio as proposed by Nimjérnahn and Ravetch (8) based on studies in the rodent system, as it seems to play a fundamental role in the evolution and function of human effector function.

Some of our findings are in contradiction with an earlier study from Jacobsen et al. (20) showing that cynomolgus IgG1 displayed the highest binding on cynomolgus monocytes compared with other human and cynomolgus IgG isotypes. In addition, cynomolgus IgG1 was far more potent in mediating ADCC in cynomolgus whole blood. The differences to the data presented in this report can be explained by the following improved technologies applied in this study. First, the size exclusion chromatography-
purified material used in our study guarantees a monomeric composition, whereas only protein A-purified material used by Jacobsen et al. (20) can result in a high percentage of aggregated, nonmonomeric Abs. For studying FcγR binding and effector function, the use of purely monomeric IgG is absolutely necessary, as FcγR shows 10- to 100-fold higher binding to aggregated IgG, and therefore, data generated with aggregated IgG would reflect the difference in the proportion of aggregated IgG rather than the mere difference in isotype selectivity. Furthermore, the affinity of IgG to the low-affinity FcγR (i.e., FcγRIIIa, FcγRIIb, and FcγRIII) is micromolar, which obscures the detection of monomeric IgG in the FACS-based assay used by Jacobsen et al. (20) due to the fast off-rate displacing the IgG from the Fc receptor during the washing steps. In contrast, the surface plasmon resonance analysis of this interaction as used in our study is able to detect the low-affinity binding and is therefore the preferable method. In addition, the effector cells (i.e., monocytes) used in the Jacobson study (20) express a variety of different FcγR, and it is therefore not possible to clearly assign to which Fc receptors the different IgG subclasses bind. The functional activity of the different IgG isoatypes was analyzed by Jacobsen et al. (20) in a whole blood B cell depletion assay using anti-CD20 Ab for depletion and CD19 as the marker for mononuclear B cells. In contrast to humans, cynomolgus monkeys show two different populations of CD20-positive B cells. Only the CD40highCD21low cynomolgus monkey B cells are depleted by rituximab similar to human B cells and in a Fc-dependent manner (29, 36) (M. Warncke, unpublished observations), and therefore we used this population for our analysis. We think that the high quality of the material used in our study together with the precise methodology allowed us to identify differences between the human and cynomolgus IgG isoatypes. The fact that the molecular sequence corresponds to the binding data and effector function further validates our approach.

Interestingly, position 131 of human FcγRIIa is also determining the high- and low-affinity binding of FcγRIIa to C-reactive protein (CRP). In contrast to IgG, CRP has a higher affinity to human FcγRIIa 131R than to human FcγRIIa 131H (37). As CRP is highly conserved in evolution and is mimicking important aspects of IgG, such as binding to FcyRs and complement (38), the discussed difference in NHP FcγRIIB may also affect the immune system beyond FcγR effector function.

Given the importance of FcγRIIB for the balance of the immune response, this remarkable shift in function of monomucous versus human FcγRIIB raises questions regarding the prediction of NHP effector function for human therapeutic Abs, as it has a direct influence on the toxicity assessment in monomucous or rhesus models of FcγRIIB-engaging agents (39, 40). For example, mutations in the Fc part of human IgG1 (S267E and L328F) led to >200-fold increased affinity to human FcγRIIB and FcγRIIa 131R, but not to FcγRIIa 131H. Thus, the key amino acid at position 131 is responsible for the increased affinity of this Fc variant (S267E and L328F) to FcγRIIB. However, this amino acid in monomucous FcγRIIB is different (Fig. 3), and therefore this mutant Fc variant (S267E and L328F) shows no enhancement in affinity to monomucous FcγRIIB (41).

In contrast to the differences identified in the silent IgG subclasses and FcγRIIb, the activating FcγR (FcγRI, FcγRIIA, FcγRIIIa) and the active IgG1 and IgG3 subclasses have comparable effector function in the human and the cynomolgus system: the binding characteristics of human and cynomolgus IgG1 and IgG3 toward human and cynomolgus FcγRs are comparable. IgG1 and IgG3 ADCC activity, which is mainly triggered in vitro by the ITAM-bearing receptor FcγRIIIa on NK cells (and to a lower extent FcγRIIA and FcγRI), and is largely independent on FcγRIIb, is indistinguishable whether human or cynomolgus effector cells are used. We therefore conclude that the observed species differences can be explained by key differences in the amino acid sequence composition at critical residues of the IgG2 and IgG4 Ab subclasses and the inhibitory receptor FcγRIIB in human and cynomolgus monkey. On the contrary, the activity profiles of the various activating FcγRs are by far and large functionally conserved between the two species. Thus, human therapeutic Abs with a wild-type IgG1 C region are expected to have a very similar effector profile in the cynomolgus monkey as compared with humans, which qualifies the cynomolgus monkey as a relevant model for this isotype. However, modifications to the IgG1 wild type, resulting in decreased or enhanced binding (24, 42–44) to FcγRIII or FcγRIIa, which are increasingly being evaluated for therapeutic use, have to be evaluated very carefully, as the difference in FcγRIII and FcγRI expression on human and cynomolgus granulocytes could influence their overall functional behavior (45). In particular, when evaluating Fc modifications altering the binding to FcγRIIB (39, 40), equivalent FcγRIIb cross-reactivity for the selected animal model species is required to allow prediction to humans.

In summary, our findings shed new light on the evolution and plasticity of IgG effector function and allow a better extrapolation of human therapeutic Ab Fc-mediated functions from the NHP to humans.

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