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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 on 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 FcγRIIIb 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 antiparasite 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 (GLNDIFEQEK nieWHE, Avidity), expressed in 293 cells and purified with anti-4APP affinity chromatography. The human receptors were expressed with a CD33 signal leader (P20138, 1-16) instead of the natural leader sequence to enhance protein expression. Receptor proteins expressed with the natural leader and the CD33 leader showed identical amino acid sequences as analyzed by mass spectrometry. The following receptor sequences were synthesized by Genentech: human FcγRIIA (CD16a)158V (Uniprot: P08637, 17-199, aa 158 = V), human FcγRIIA 158F (Uniprot: P08637, 17-199), cynomolgus FcγRIIA, human FcγRIIA (CD32a) 131H (Uniprot: NP_067674, 34-217), human FcγRIIA 131R (Uniprot: NP_067674, 34-217, aa 131 = R), cynomolgus FcγRIIA (Uniprot: AAL92096, 1-210), human FcγRIIB (CD32b) (Uniprot: P31994, 42-223), and cynomolgus FcγRIIB (Uniprot: AAL92097, 1-226). Receptors were site directed biotinylated with BirA (Avidity). Extracellular domain of human FcγRI (CD64) 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′-ATCCAAAGCAGCTGTGGCTGACA-CCA-3′, 5′-ATTAACACCTCTCCTCCTTGA-3′; γ H chain, 5′-GGCTCTCACAACGGGCCCATCAGCCGAGAG-3′). Genomic DNA from the blood of 10 cynomolgus monkeys was sequenced to validate the γ H chain C regions (primers: 5′-GGCCCGACTCTGTCACCACCGC-3′, 5′-CCTGGGAAGTATGTACACGG-3′). Human and cynomolgus κ L chain C regions and cynomolgus IgG3 were synthesized by Geneart, Uniprot accession numbers of human IgG1-4: P01857, P01859, P01860, and P01861; human κ L chain: P01834; cynomolgus IgG3, GenBank: D30779 (18). GenBank database: http://www.ncbi.nlm.nih.gov/nuccore. Human and cynomolgus H and L chain C regions were cloned directly after the anti-CD20 mAb Rituximab (19) or the anti-CD28 mAb TGN1412 (14) V region, mouse or human IgG2, κ L chain, 5′-ATCAAACGAGCTGTGGCTGCA-3′, 5′-ATTAACACTCTCCCCTGTTGA-3′; mouse IgG2b, κ L chain, 5′-ATCAAACGAGCTGTGGCTGCA-3′, 5′-ATTAACACTCTCCCCTGTTGA-3′; mouse IgG2a, κ L chain, 5′-ATCAAACGAGCTGTGGCTGCA-3′, 5′-ATTAACACTCTCCCCTGTTGA-3′; mouse IgE, κ L chain, 5′-ATCAAACGAGCTGTGGCTGCA-3′, 5′-ATTAACACTCTCCCCTGTTGA-3′. Functional assays

ADCC activity was assessed by the depletion of B cells. PBMCs were purified by Ficoll gradient, 100% Ficoll was used for the human blood separation, and 90% Ficoll plus 10% PBS for the cynomolgus blood separation. Human or cynomolgus PBMC were incubated for 1 h with the anti-CD20 Abs, stained for B cells with anti-CD40 (5C3; BioLegend) and anti-CD21 (B-ly4, BD Biosciences), and analyzed by flow cytometry. Killing was calculated as percentage of B cells without Ab to percentage of B cells with Ab.

Results

Different FcγR 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 FcγRIIIA, 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 Vi, derived from the CD20-binding Ab rituximab (19) recombinantly expressed with the four human and cynomolgus IgG subclasses were used to...
and CH2 domain that dramatically decrease their affinity for can be explained by differences to IgG1 in residues of the hinge (Fig. 3). The "silent" effector function of human IgG2 and IgG4 account for the different affinity of cynomolgus IgG2 and IgG4 with a 5–10 higher affinity human and cynomolgus Fc

3

cynomolgus Fc

g has a higher affinity than Fc

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IgG2 and IgG4 (Fig. 2A, 2B, Supplemental Figs. 2, 3, Table I). Distinct amino acid substitutions in the phenotype (Table I). Different amino acid substitutions in the cynomolgus IgG sequences at the Fc

g

RIII-158F (23). Interestingly, cynomolgus Fc

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RIII 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

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RIII binding domains (24, 25) account for the different affinity of cynomolgus IgG2 and IgG4 (Fig. 3). The "silent" effector function of human IgG2 and IgG4 can be explained by differences to IgG1 in residues of the hinge and Cγ2 domain that dramatically decrease their affinity for FcγRs and complement, therefore rendering them inactive for ADCC or CDC (26). This is not the case for cynomolgus IgG2 and IgG4, where these residues are identical to the full effector function bearing IgG1 and IgG3 isotypes (Fig. 3A).

High-affinity binding of cynomolgus FcγRIIb to IgG2

Interestingly, all the activating ITAM-bearing FcγRs of human and cynomolgus origin show almost identical binding profiles. One striking difference is that the cynomolgus inhibitory FcγRIIb binds IgG2 with substantially higher affinity than human FcγRIIb (Fig. 2C). This difference in binding is directly correlated with a different amino acid pattern in the Fc binding region of cynomolgus FcγRIIb (Fig. 3C). There is a polymorphism in the Fc binding region of the activating human FcγRIIa, which has a higher affinity to IgG2 if the amino acid at position 131 is a histidine. Accordingly, human FcγRIIa-131H and cynomolgus FcγRIIa (131H) show a higher affinity to IgG2 than human FcγRIIa 131R (Fig. 2C). In contrast to human FcγRIIb, in which the amino acid at position 131 is an arginine leading to low-affinity binding of IgG2, cynomolgus FcγRIIb 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...
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 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 C3 domain of cynomolgus IgG4 seems to be important for Fab arm exchange, because the exchange of this domain by the human or rhesus C3 complete 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/human IgG4 Abs generated a third mass exchange, because the exchange of this domain by the human or rhesus CH3 completely restored the Fab arm exchange. The human or rhesus CH3 completely restored the Fab arm exchange. Because the exchange of this domain by the human or rhesus CH3 completely restored the Fab arm exchange. 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 of 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., CD20lowCD40highCD21+ and CD20highCD40lowCD21+). Because the CD20lowCD40highCD21+ 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 CD40highCD21+ 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). The C3 domain of cynomolgus IgG4 seems to be important for Fab arm exchange, because the exchange of this domain by the human or rhesus C3 completely restored the Fab arm exchange with human IgG4 (Fig. 4B). 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).

**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-
that recombinant Abs with rhesus IgG4 C regions display similar or even increased IgG4 Fab arm exchange than human IgG4. It has also been described that human IgG4 therapeutic Abs can perform Fab arm exchange with cynomolgus serum Abs, but the IgG4 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-affinity 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 monkey 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 FcγRI, FcγRIIb, supports the model of activating-to-inhibitory ratio as proposed by Nimmerjahn 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-

FIGURE 4. Fab arm exchange of TGN1412 with cynomolgus IgG4. (A) A total of 100 μg human rituximab-IgG4 and human TGN1412 (solid line), or 100 μg human rituximab-IgG4 (dashed line) and human TGN1412, or 100 μg human rituximab-IgG4 alone (gray filled histogram) was injected into SCID mice (n = 2), and Fab arm exchange was analyzed 48 h later. (B) A total of 1 μg human rituximab-IgG4 was incubated for 24 h at 37°C with different IgG4 variants of TGN1412 in the presence of GSH. The TGN1412 variants were constructed by exchanging the human IgG4 C region by rhesus IgG4, cynomolgus IgG4 (wild type), cynomolgus IgG4 S131C, cynomolgus IgG4 P228S, cynomolgus IgG4 with a human Cγ3 domain, and cynomolgus IgG4 with a rhesus Cγ3 domain. (C–F) LC-MS analysis of IgG4 Fab arm exchange. A total of 100 μg each Ab was incubated for 24 h at 37°C in 10 μl PBS and analyzed by LC-MS: (C) human rituximab-IgG4 + human TGN1412 without GSH; (D) human rituximab-IgG4 + human TGN1412 in the presence of GSH; (E) human rituximab-IgG4 + cynomolgus TGN1412 S131C without GSH; (F) human rituximab-IgG4 + cynomolgus TGN1412 S131C in the presence of GSH.

molgus granulocytes, consisting of neutrophils and eosinophils, do not express FcγRIIb, but instead display FcγRI at very high levels. Although FcγRIIb is a GTP-linked receptor, it still supports a variety of signaling events and is thought to enhance the overall avidity of IgG binding, thereby contributing to the IgG subclass specificity and FcγR-mediated function of human granulocytes (30, 31). Bacteria are mainly opsonized by carbohydrate-specific IgG2 subclass Abs (16, 17), whereas helminth parasites and allergens preferentially induce the generation of IgG4 (32, 33). Strikingly, only IgG2 and IgG4 properties are different in cynomolgus monkey, demonstrating potent FcγR binding and effector function, whereas IgG1 and IgG3 subclasses show an almost identical profile in both cynomolgus monkeys and humans. The potential effector function of cynomolgus IgG4 is further increased by mutations in the hinge and Cγ3 abrogating Fab arm exchange. Whether this is a general feature of all cynomolgus monkey subspecies and/or from different origins will require further investigation. This is in particular relevant, because, in the rhesus monkey, the critical amino acids involved in Fab arm exchange display allotypic variations (18, 34), and we could show
purified material used in our study guarantees a monomeric composition, whereas only protein Α-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., FcγRIIa, FcγRIIb, and FcγRIIIa) 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 subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. J. Exp. Med. 145: 1316–1327.

Given the importance of FcγRIIb for the balance of the immune response, this remarkable shift in function of cynomolgus 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 cynomolgus 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 cynomolgus FcγRIib is different (Fig. 3), and therefore this mutant Fc variant (S267E and L328F) shows no enhancement in affinity to cynomolgus FcγRIib (41).

In contrast to the differences identified in the silent IgG subclasses and FcγRIib, the activating FcγR (FcγRI, FcγRIIA, FcγRIIb) 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 FcγRIIa 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 FcγRIIb or FcγRIIA, which are increasingly being evaluated for therapeutic use, have to be evaluated very carefully, as the difference in FcγRII 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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Disclosures

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