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

Max Warncke, Thomas Calzascia, Michele Coulot, Nicole Balke, Ratiba Touil, Frank Kolbinger and Christoph Heusser

*J Immunol* published online 28 March 2012
http://www.jimmunol.org/content/early/2012/03/28/jimmunol.1200090

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/03/28/jimmunol.1200090.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Different Adaptations of IgG Effector Function in Human and Nonhuman Primates and Implications for Therapeutic Antibody Treatment

Max Warncke, Thomas Calzascia, Michele Coulot, Nicole Balke, Ratiba Touil, Frank Kolbinger, and Christoph Heusser

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 FcγRIIb, but show higher levels of FcγRIIb 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.

The complex interplay of Fc receptors and Abs has been an area of scientific interest for the last decades. More than 30 years ago, we and others could show that different IgG Fc receptors (FcγR) exist that are highly selective in terms of subclass specificity and affinity (1, 2). The high complexity of this system is increased by the existence of activating and inhibitory FcγRs (3) and the fact that the different effector cell populations are decorated with different types and different densities of Fc receptors (4). Interaction of monomeric Abs or Ab immune complexes with Fc receptors triggers Ab-dependent cellular cytotoxicity (ADCC) and Ab-dependent cellular phagocytosis, whereas interaction with complement results in complement-dependent cytotoxicity (CDC) (5). From a signaling standpoint, activation of the cellular effector functions is mediated by ITAM-containing FcγRs (CD32b), which plays a critical role in modulating IgG-immune complexes (e.g., opsonized bacteria or parasites). These activities are counterregulated by the ITIM-containing FcγRIIB (CD32b), which plays a critical role in modulating IgG-mediated effector functions, such as in humoral immunity (6, 7). The relative densities of inhibitory (i.e., FcγRIIB) versus activating FcγRs, and in particular their corresponding selectivity and affinity toward different IgG isotypes, determine whether an Ab will generate an activating proinflammatory or an inhibitory anti-inflammatory response. For a given IgG isotype, Ravetch and Nimmerjahn (8) have found that the ratio of affinities of the activating ITAM-bearing FcγRs to the inhibitory FcγRIIB is a key determining factor. In the case of therapeutic Abs, IgG/FcγR interactions contribute significantly to both therapeutic effect and toxicity of these drugs (5). Due to their proximal evolutionary relationship to humans, the most appropriate preclinical model to analyze the therapeutic effect and potential toxicity of human Abs are nonhuman primates (NHP), in particular the cynomolgus monkey (9). However, recent clinical trial results have questioned whether NHP are an appropriate model. A striking example is the outcome of the TGN1412 trial in 2006, in which an anti-CD28 Ab (TGN1412) did not show severe adverse side effects in NHP, but generated a dramatic cytokine storm in all six treated human subjects (10). Possible contributions to this differential response in NHP and humans in this trial include differences in the signaling domain of CD28, the loss of Siglec-5 expression during human evolution (11, 12), or a different expression pattern of CD28 on a specific T cell subset (13). However, because triggering of a cytokine storm by TGN1412 is dependent on FcγR interaction (14), it has also been speculated that the human IgG4 Fc portion of the TGN1412 Ab might bind less efficiently to monkey than to human FcγRs (15).

In light of the growing use of IgG mAb as biotherapeutic compounds, we further investigated the interaction of human and cynomolgus IgG subtypes and FcγRs to assess the appropriateness of cynomolgus monkeys in evaluating Fc-dependent efficacy and toxicity profiles of therapeutic IgGs. Our study revealed that the effector function of humans and NHP is an adaptable system, which is shaped by species-specific constraints. We have found clear differences in FcγR expression on granulocytes, allotypic variation of IgG4 Fab arm exchange, and enhanced activity of IgG2 and IgG4 subclasses in cynomolgus monkeys compared with the human system, which may have been evolved by different needs in phagocytosis and neutralization of bacteria and parasites. Interestingly, this overall enhanced activation function in cynomolgus monkeys is counterbalanced by a stronger inhibitory capacity of cynomolgus FcγRIIB toward IgG2, an isotype with preference for repetitive carbohydrate Ags on bacteria (16, 17),...
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 FcγRI/II/III 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 (GLNDIFEAQKIEWHE; Avidity), expressed in 293 cells and purified with anti-4APP affinity chromatography. The human receptors were expressed with a CD33 signal leader (P0138, 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 Geneart: human FcγRIIA (CD16a) 158V (Uniprot: P08637, 17-199, aa 158 = V), human FcγRIIB (CD18a) 131H (Uniprot: NP_067674, 34-217), human FcγRIIA 131R (Uniprot: NP_067674, 34-217, aa 131 = R), cynomolgus FcγRIIB (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 domains of human FcγRI (CD64) were purchased from R&D Systems (Minneapolis, MN). Cynomolgus κ L chain (GenBank: JN984930) and γ H chain C regions (IgG1, GenBank: JN984927; IgG2, GenBank: JN984927; IgG4, GenBank: JN984928) were cloned from cynomolgus blood mRNA (primer κ L chain, 5'-ATCAGACAGGCTTGGCTGCA- CCA-3', 5'-ATTAACACTCCTCTCTTGTA-3', γ H chain, 5'-GCTCCT- CACCAAGGCGCATCAGG-3', 5'-TTTACCCGGAGACGGAGAG-3'). Genomic DNA from the blood of 10 cynomolgus monkeys was sequenced to validate the γ H chain C regions (primer: 5'-GGCCGACCTGCTGCC- CACACC-3', 5'-CCTGGGAAATGTATGACCGG-3'). Human κ and L chain C regions and cynomolgus IgG3 were synthesized by Geneart, Uniprot accession numbers of human IgG1-κ: P01837, P01859, P01860, and P01861; human κ L chain: P01834; cynomolgus IgG3, GenBank: DQ617798 (5), 3D3-FITC, and FLI8.26-FITC. Uniprot database: http://www.uniprot.org/uniprot/. Gen- Bank 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, expressed in 293 cells, and purified by protein G and size exclusion chromatography to a purity of >99% monomeric IgG. There was no sign of reaggregation during the course of the study, as assessed by analytical size exclusion chromatography. Analysis of the protein size under reducing and nonreducing conditions revealed human IgG4 half-molecule formation and cynomolgus IgG4 κ-H-L chain dimer formation similar to a recent report (20), and all Abs showed comparable binding to CD20-expressing cells (Supplemental Fig. 1).

**CD32a allotyping**

The 100-μl samples of whole blood were incubated for 30 min at 4°C in the dark with each of the FITC-conjugated Abs (dansyl-specific isotype control Ab clone 27.18; mouse IgG2b, κ), 3D3-FITC, and FLI8.26-FITC [BD Biosciences]). After RBC lysis, cells were washed and analyzed by flow cytometry. Analysis was restricted to granulocytes gated by their forward light scatter versus side light scatter. The ratio of 3D3 minus isotype control geometric mean fluorescence intensity (ΔGMI) versus FLI8.26 ΔGMI was used to determine the genotype of the different donors. Ratios are as follows: heterozygous donors, ~0.5; CD32aκ+/κ+ homozygous donors, ~1; CD32aκ+/ω+ homozogous donors, ~2. For FcγRII, the Rituximab-derived Abs were captured by an anti-

**Rituximab idiotypic Ab** (2842; AbD Serotec), and the binding to human and cynomolgus FcγRI was analyzed. Association (ka) and dissociation (kd) rate constants were calculated by a 1:1 model; Kd was calculated as kd/ka. The reciprocal value for the equilibrium dissociation constant, was calculated as 1/Kd.

**IgG4 Fab arm exchange**

For in vivo Fab arm exchange, 100 μg human rituximab-IgG4 and human TGN1412, or 100 μg cynomolgus rituximab-IgG4 and human TGN1412, or 100 μg human rituximab-IgG4 alone was injected into SCID mice, and Fab arm exchange was analyzed 48 h later. For in vitro Fab arm exchange, 1 μg human rituximab IgG4 was incubated for 24 h at 37°C with 1 μg of the different IgG4 variants of TGN1412 in the presence of 0.5 mM reduced glutathione (GSN). To analyze the generation of bispecific Abs, CD28-expressing Jurkat cells or CD20-expressing Raji cells were incubated with the serum of the injected animals or the Ab mixtures and stained with fluorescent anti-rituximab idiotype-specific Ab (MB2 A4; AbD Serotec) or CD28-Fc. To analyze the generation of a third Ab species with mass corresponding to a H-L chain dimer of each parental Ab, liquid chromatography-mass spectrometry (LC-MS) (Waters Acquity UPLC system coupled directly to a Waters Q TOF Premier Mass Spectrometer) analysis of PNGase F (New England Biolabs)-treated Ab mixture was performed. The resulting N-deglycosylated sample was injected on a MassPrep Micro desalting column (2.1 × 5 mm; Waters) heated at 80°C at a flow rate of 0.4 ml/min with a gradient of 2–90% solvent B. Mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: acetonitrile with 0.1% formic acid. The electrospray ionization time-of-flight mass spectrometer was operated in the positive V-mode, under a source temperature of 120°C, a desolvation temperature of 250°C, a sample cone set at 40 V, and a capillary voltage of 1.5 kV. Mass spectra were acquired in the m/z range of 1000–6000. A 0.95-Hz scan rate with 0.05-s interscan delay was used to acquire data over the entire analysis. The deconvoluted electrospray ionization time-of-flight spectra using MaxEnt1 (Waters) are shown.

**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 FcγRIIa, and therefore become highly activated by IgG-opsonized bacteria or parasites. In contrast to humans, NHP do not express any form of FcγRII 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 FcγRII has a different binding profile in comparison with FcγRI, 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
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 cynomolgus Fc

has a higher affinity than Fc

g

IgG2 and IgG4 with a 5–10
cynomolgus IgG sequences at the Fc

phenotype (Table I). Distinct amino acid substitutions in the isoleucine at position 158 and accordingly shows a high-affinity high-affinity binding of cynomolgus Fc

RIII and Fc

RI in comparison with their human Rs 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). In summary, cynomolgus IgG2 and IgG4 show increased binding to human and cynomolgus activating receptors FcγRI and FcγRIII, cynomolgus IgG2 and IgG4 showed substantial binding to corresponding FcγR of both species (Fig. 2). Human and cynomolgus FcγRI 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 has 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 FcγR 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). In summary, cynomolgus IgG2 and IgG4 show increased binding to human and cynomolgus FcγRIII and FcγRI in comparison with their human counterparts, whereas human and cynomolgus IgG1 and IgG3 show an almost identical binding to human and cynomolgus FcγRIII and FcγRI.

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

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 FcγR. (A–G) Binding of the different isotypes to (A) human FcγRI, equilibrium-binding constants (Kₐ); (B) human FcγRI, dissociation rates (k_d); (C) FcγRIIa, Kₐ, and FcγRIIb; (D–G) binding levels at equilibrium: (D, E) human FcγRIIa; (F, G) cynomolgus FcγRIIa. 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.
human primate, and NHP Fc
of the human parental Abs. In combinations of human/human IgG4 Abs generated a third mass ex-
cysteine-containing version of cynomolgus IgG4 (S131C). Com-
method, we used LC-MS under denaturing conditions and the exchange with human IgG4 (Fig. 4B). To demonstrate the gen-
portant for Fab arm exchange, because the exchange of this do-
Molecular sequence of human and cynomolgus IgG and
contrast, the CH3 domain of cynomolgus IgG4 seems to be im-
ike hinge region (P238S) did not increase Fab arm exchange. In human IgG4, and thus, the H and L chains are only held together
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 mon-
kons 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 ex-
change (Fig. 4B). Notably, cynomolgus IgG4 with a human IgG4-like hinge region (P238S) did not increase Fab arm exchange. In contrast, the Cγ3 domain of cynomolgus IgG4 seems to be im-
portant for Fab arm exchange, because the exchange of this do-
momain by the human or rhesus Cγ3 completely restored the Fab arm exchange with human IgG4 (Fig. 4B). To demonstrate the gen-
eration 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). Com-
binations of human/human IgG4 Abs generated a third mass ex-
ctly between the masses of the two human parental Abs.

Table I. Overview of binding affinities to FcγR and ADCC potency of the different anti-CD20–based human and cynomolgus isotype Abs

<table>
<thead>
<tr>
<th>Cellular binding (n = 2)</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>
</tr>
</thead>
<tbody>
<tr>
<td>Cyto FcyRI 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>
</tr>
<tr>
<td>Biacore binding (n = 2)</td>
<td>Human FcyRI Kp (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>
</tr>
<tr>
<td>Human FcyRLI EC50 (nM)</td>
<td>0.37 ± 0.03</td>
<td>17 ± 0.2</td>
<td>0.42 ± 0.02</td>
<td>13.3 ± 0.4</td>
<td>0.46 ± 0.1</td>
<td>3.32 ± 0.15</td>
<td>0.62 ± 0.03</td>
<td>2.87 ± 0.06</td>
</tr>
<tr>
<td>Human FcyRLIII EC50</td>
<td>1.71 ± 0.1</td>
<td>49 ± 6</td>
<td>1.47 ± 0.06</td>
<td>42 ± 4</td>
<td>1.12 ± 0.07</td>
<td>5.98 ± 0.04</td>
<td>1.79 ± 0.02</td>
<td>7.7 ± 0.56</td>
</tr>
<tr>
<td>Cyto FcyRIII</td>
<td>0.36 ± 0.01</td>
<td>11 ± 0.8</td>
<td>0.4 ± 0.04</td>
<td>11 ± 2.4</td>
<td>0.48 ± 0.01</td>
<td>2.38 ± 0.07</td>
<td>0.62 ± 0.02</td>
<td>2.84 ± 0.02</td>
</tr>
<tr>
<td>Human FcyRII A 158V</td>
<td>0.56 ± 0</td>
<td>0.37 ± 0.06</td>
<td>1.69 ± 0.02</td>
<td>4.01 ± 0.29</td>
<td>0.28 ± 0.03</td>
<td>1.13 ± 0.31</td>
<td>0.10 ± 0.01</td>
<td>2.2 ± 0.13</td>
</tr>
<tr>
<td>Human FcyRII A 131H</td>
<td>1.12 ± 0.11</td>
<td>2.18 ± 0.21</td>
<td>2.1 ± 0.16</td>
<td>3.23 ± 0.1</td>
<td>0.43 ± 0.02</td>
<td>6.2 ± 0.2</td>
<td>0.45 ± 0.04</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>Human FcyRII B</td>
<td>1.78 ± 0.06</td>
<td>0.88 ± 0.02</td>
<td>2.36 ± 0.15</td>
<td>4.55 ± 0.02</td>
<td>1.07 ± 0.04</td>
<td>2.13 ± 0.04</td>
<td>1.94 ± 0.01</td>
<td>2.72 ± 0.13</td>
</tr>
<tr>
<td>Human FcyRLII</td>
<td>4.88 ± 0.02</td>
<td>10.89 ± 0.1</td>
<td>6.5 ± 0.2</td>
<td>6.5 ± 0.2</td>
<td>3.75 ± 0.14</td>
<td>2.08 ± 0.05</td>
<td>19.7 ± 0.8</td>
<td>3.71 ± 0.09</td>
</tr>
<tr>
<td>Human FcyRLIIb</td>
<td>1.84 ± 0.05</td>
<td>0.47 ± 0</td>
<td>4.21 ± 0.13</td>
<td>4.79 ± 0.1</td>
<td>1.17 ± 0.05</td>
<td>1.92 ± 0.02</td>
<td>3.59 ± 0.05</td>
<td>4.94 ± 0.34</td>
</tr>
<tr>
<td>ADCC EC50 (μg) (n = 4)</td>
<td>Human PMBC</td>
<td>0.04 ± 0.04</td>
<td>11 ± 0.9</td>
<td>0.09 ± 0.10</td>
<td>3.6 ± 4.9</td>
<td>0.02 ± 0.01</td>
<td>0.16 ± 0.05</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Cyto PMBC</td>
<td>0.03 ± 0.02</td>
<td>13 ± 1.0</td>
<td>0.09 ± 0.04</td>
<td>0.94 ± 0.60</td>
<td>0.05 ± 0.03</td>
<td>0.40 ± 0.20</td>
<td>0.07 ± 0.04</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

*Value is extrapolated and is outside the range of the used concentrations.

Discussion

Despite the reported sequence homology of human and cyno-
molgus 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 CYMONOLGUS IgG4 IS SHOW AN ALMOST IDENTICAL PROFILE IN BOTH CYMONOLGUS MONKEYS AND BINDING AND EFFETOR FUNCTION, WHEREAS IgG1 AND IgG3 SUBCLASSS.

AND ALLERGENS PREFERENTIALLY INDUCE THE GENERATION OF IgG4 (AND SPECIFIC IgG2 SUBCLASS ABS) (16, 17), WHEREAS HELMINTH PARASITES CONTRAST TO HUMANS AND HUMAN PRIMATES. THIS COEVOLUTION OF ACTIVATING IgG ISOTYPES AND THE INHIBITORY FcγR, FcγRIII, SUPPORTS THE MODEL OF ACTIVATING-TO-INHIBATORY 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 EFFETOR FUNCTION.

SOME OF OUR FINDINGS ARE IN CONTRACTION WITH AN EARLIER STUDY FROM JACOBSEN ET AL. (20) SHOWING THAT CYMONOLGUS IgG1 DISPLAYED THE HIGHEST BINDING ON CYMONOLGUS MONOCYTES COMPARED WITH OTHER HUMAN AND CYMONOLGUS IgG ISOTYPES. IN ADDITION, CYMONOLGUS IgG1 WAS FAR MORE POTENT IN MEDIATING ADCC IN CYMONOLGUS WHOLE BLOOD. THE DIFFERENCES TO THE DATA PRESENTED IN THIS STUDY (35).

OF NOTE, WE FOUND THAT THE INCREASE OF ACTIVATING FUNCTION OF CYMONOLGUS IgG2 (I.E., HIGHER BINDING TO FcγRI AND FcγRIII AND HIGHER EFFETOR FUNCTION) IS COUNTERBALANCED BY A STRONGLY INCREASED AFFINITY TOWARDS CYMONOLGUS FcγRIIb. INTERESTINGLY, IN CONTRAST TO FcγRIIb IN THE NHP MACAQUE FAMILY MEMBERS CYMONOLGUS AND RHEUS 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 TOWARDS 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 COEVOLED AND THAT MACAQUE SPECIES SUCH AS THE CYMONOLGUS AND RHEUS MONKEY HAVE DEVELOPED A DIFFERENT STRATEGY TO BALANCE IgG/FcyR INTERACTIONS IN CONTRAST TO HUMANS AND HUMAN PRIMATES. THIS COEVOLUTION OF ACTIVATING IgG ISOTYPES AND THE INHIBITORY FcγR, FcγRIII, SUPPORTS THE MODEL OF ACTIVATING-TO-INHIBATORY 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 EFFETOR FUNCTION.

SOME OF THE FINDINGS ARE IN CONTRACTION WITH AN EARLIER STUDY FROM JACOBSEN ET AL. (20) SHOWING THAT CYMONOLGUS IgG1 DISPLAYED THE HIGHEST BINDING ON CYMONOLGUS MONOCYTES COMPARED WITH OTHER HUMAN AND CYMONOLGUS IgG ISOTYPES. IN ADDITION, CYMONOLGUS IgG1 WAS FAR MORE POTENT IN MEDIATING ADCC IN CYMONOLGUS 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., FcγRIIa, 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 cytomolgus B cells. In contrast to humans, cytomolgus monkeys show two different populations of CD20-positive B cells. Only the CD40highCD21+ 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 cytomolgus IgG isotypes. 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, FcγRIIIa than to human FcγRIIb (37). As CRP is highly conserved in evolution and is mimicking important aspects of IgG, such as binding to FcγRs 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 cytomolgus 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 cytomolgus 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 cytomolgus FcγRIIb is different (Fig. 3), and therefore this mutant Fc variant (S267E and L328F) shows no enhancement in affinity to cytomolgus 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 cytomolgus system: the binding characteristics of human and cytomolgus IgG1 and IgG3 toward human and cytomolgus 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 FcγRIla and FcγRI), and is largely independent on FcγRIIb, is indistinguishable whether human or cytomolgus 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 cytomolgus monkey. On the contrary, the activity profiles of the various activating FcγRs are by 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 cytomolgus monkey as compared with humans, which qualifies the cytomolgus 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 FcγRIIIa, which are increasingly being evaluated for therapeutic use, have to be evaluated very carefully, as the difference in FcγRIIb and FcγRIIIa expression on human and cytomolgus granulocytes could influence their overall functional behavior (45). In particular, when evaluating Fc modifications altering the binding to FcγRIIIa (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.

Acknowledgments
We thank Holger Heine, Patrick Schindler, Stefan Ewart, Steffen Hartmann, Cornelius Fritsch, and Thomas Pietzonka for helpful discussion and David Lee for critical comments on the manuscript.

Disclosures
All authors are employees and/or shareholders of Novartis.

References


Supplemental Data:

**Supplementary Figure 1:** Molecular and biochemical properties of recombinant human and cynomolgus IgG

A) Non-reduced and reduced SDS electrophoresis of Rituximab-based antibodies. In the non-reduced state human IgG4 shows an additional band at the size of a heavy-light chain molecule (HL), cynomolgus IgG4 show two additional bands at the size of a heavy-chain dimer (H2) and a light chain dimer (L2) molecule. In the reduced state all molecules show the expected heavy (H)
and light chain (L) monomers B) Affinity competition assay of Rituximab-basaed antibodies. The CD20 expressing Ramos cell line was incubated with a fluorescent labeled Rituximab (human IgG1) and different concentrations of the isotype switched Rituximab-based variants. Competition binding was analyzed by flow cytometry and fitted to log(inhibitor) vs. response equation (Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) (n=2).
Supplementary Figure 2: Biacore binding analysis of captured Rituximab-based human and cynomolgus IgG isotypes to soluble human FCγR1. Rituximab-based antibodies were captured with a covalently coupled anti idiotypic antibody and the binding to different concentration of recombinant soluble FCγR1 was analyzed. Please note that the reduced $R_{\text{max}}$ of human IgG3 is
caused by the lower affinity of the anti-idiotypic antibody to human IgG3 and the affinity and kinetic of the IgG-FCγR1 interaction is not affected. On-rate and off-rate were simultaneously fitted by a 1:1 model. (n=2).

Supplementary Figure 3: Binding analysis of Rituximab-based human and cynomolgus IgG isotypes to a cynomolgus FCγR1 stable transfected CHO cell line. Different concentrations of Rituximab-based antibodies were incubated with CHO-cynomolgus FCγR1 and detected with a FITC-coupled anti-idiotypic antibody. Binding levels were analyzed by flow cytometry and fitted to a saturation binding equation (Y = Bmax*X/(Kd + X)) (n=2).
Supplementary Figure 4: CDC effector function of human and cynomolgus IgG isotypes. Calcein-AM stained human B cell line Ramos was incubated with different concentrations of Rituximab-based antibodies in the presence of human serum. After 1h calcein release in the supernatant was analyzed. Triton X-lysed Ramos cells (maximal lysis) and Ramos cells alone (blank) were served as controls. Data were normalized to maximal lysis and blank and fit to a sigmoidal dose-response model (n=2).