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Specificity of mouse and human Fcγ receptors and their polymorphic variants for IgG subclasses of different species

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Abstract

Immunoglobulin G (IgG) is the predominant antibody class generated during infections and used for the generation of therapeutic antibodies. Antibodies are mainly characterized in or generated from animal models that support particular infections, respond to particular antigens or allow the generation of hybridomas. Due to the availability of numerous transgenic mouse models and the ease of performing bioassays with human blood cells *in vitro*, most antibodies from species other than mice and humans are tested *in vitro* using human cells and/or *in vivo* using mice. In this process, it is expected, but not yet systematically documented, that IgG from these species interact with human or mouse IgG receptors (FcγRs). In this study, we undertook a systematic assessment of binding specificities of IgG from various species to the families of mouse and human FcγRs, including their polymorphic variants. Our results document the specific binding patterns for each of these IgG (sub)classes, reveal possible caveats of antibody-based immunoassays, and will be a useful reference for the transition from one animal model to preclinical mouse models or human cell-based bioassays.

Introduction

Different animal models are used to study various aspects of immunity and in particular antibody-driven functions. Therapeutic antibodies are often evaluated for efficacy and toxicity in a different animal species from which they originated. Rodents and non-human primates are primary models for preclinical testing and infection studies, because they share similarities to humans regarding symptoms and triggered immune responses. Therapeutic antibodies are essentially of the IgG antibody class that exert many of their biological functions through the crystallizable fragment (Fc) that can engage IgG receptors (FcγRs) and complement[1]. Transfer of IgG from various species into validated *in vitro* bioassays involving human cells or into transgenic strains of mice for *in vivo* studies is tempting, but requires knowledge on IgG-FcγR interactions. In addition, IgGs from various

species are used as reagents for routine experimental procedures and notably immunostainings that may be affected by IgG-Fc γ R interactions. In humans four IgG subclasses exist that all have specific binding profiles to the six human Fc γ Rs and their polymorphic variants[2]. Non-human primate IgG equally express four IgG subclasses, but their sequences and structures differ from human IgG subclasses[3, 4]. Mice produce four IgG subclasses (IgG1, IgG2a/c, IgG2b, IgG3) and express four classical IgG receptors. While binding of IgG to Fc γ Rs within a given species is rather well-documented[2, 5-7], only a few studies investigated binding specificities across species using various assays[8-13]. We therefore undertook herein to describe the interaction of IgG (sub)classes from twelve different species to human and mouse Fc γ Rs.

Results and Discussion

The majority of human and mouse Fc γ Rs display low affinities for human and mouse IgG, precluding detectable interactions with IgG monomers. They do, however, readily interact with IgG complexes, either immune complexes (ICs) made of antigen and antigen-specific IgG, or complexes made of anti-IgG F(ab')₂ fragments and IgG (termed Fab₂Cs herein)[14]. We used a collection of CHO transfectants[2, 15] expressing a single Flag-tagged human or mouse Fc γ R to evaluate the binding of Ig complexes from different species, including human, cynomolgus monkey, mouse, rat, hamster, rabbit, guinea pig, cow, horse, sheep and goat IgG, and also chicken IgY. Binding of Ig complexes was assessed by incubation of the CHO cell collection with fluorescent Fab₂Cs. In addition, for mouse, human, and rabbit IgG by incubation with preformed ICs made of anti-trinitrophenyl (TNP) IgG switch-variants or hybridomas and biotinylated TNP-labeled BSA. Non-transfected CHO cells were included in each experiment to monitor unspecific binding.

IgG binding to mouse Fc γ Rs

Interactions of *mouse* IgG subclasses with mouse Fc γ R using both types of IgG complexes (Figures 1C/H, S1B) reproduced the pattern previously described[6, 7]. Mouse IgG1 bound to mFc γ RIIB and mFc γ RIII, mouse IgG2a and IgG2c bound to all mFc γ Rs, mouse IgG2b to mFc γ RIIB, mFc γ RIII and mFc γ RIV, and mouse IgG3 to mFc γ RI[14, 16]. Unexpectedly, mIgG3 complexes bound detectably to mFc γ RIII when present in form of an IC and exhibited weak binding to mFc γ RIV when present as Fab₂C.

Among *human* IgG subclasses, IgG1, IgG3 and IgG4 bound all mFc γ Rs, whereas binding of human IgG2 to mFc γ RIIB, mFc γ RIII and faintly to mFc γ RIV was only detectable using ICs (Figures 1D/I, S1C). Our data reveal broader cross-binding towards mFc γ Rs than previously reported using either a binding competition assay[8] that might require stronger IgG-Fc γ R interactions to be revealed, or surface plasmon resonance that relies on monomeric interactions between human IgG and mFc γ Rs[9, 12]. Of note, we used herein the stabilized hIgG4 S₂₂₈P variant[17] that was reported to yield detectable binding to mFc γ RIIB[12].

ICs made of any of the four *macaque* IgG subclasses or *rabbit* IgG (composed of a single IgG subclass) bound to all mFc γ Rs (Figures 1E/F/J/K, S1D/E).

Fab₂Cs made of *rat* IgG1, IgG2a, and IgG2b demonstrated distinctive patterns (Figures 1L, S1G), with rat IgG2b complexes binding to all mFc γ Rs[18], rat IgG1 complexes exclusively to mFc γ RIIB and mFc γ RIII, and rat IgG2a complexes not binding mFc γ Rs, with the exception of a weak interaction with mFc γ RIII. This difference may explain why cell type depleting rat IgG2b antibodies work much more efficiently in mice than rat IgG2a[19].

Syrian (S) or Armenian (A) *hamster* IgG subclasses are still ill-defined[20]. Total hamster IgG (S/A) and IgG1 (A) showed binding to mFc γ RIII and weakly to mFc γ RIIB, whereas hamster “IgG3” (A) failed to bind significantly to any mFc γ R (Figures 1M, S1H). These results confirm the observation that the Armenian hamster IgG1 anti-mFc γ RIV mAb 9E9 may also block Fc γ RIII dependent activities at least under certain experimental conditions [21, 22].

Other species could only be tested using a pool of total IgG, e.g. horse - 7 IgG subclasses[23], ruminants - 3 IgG subclasses[24], guinea pig - 1 or 2 IgG subclasses[25, 26]. Guinea pig IgG complexes bound to all mFcγRs; sheep IgG complexes bound only to mFcγRIII; goat IgG complexes only to mFcγRIII and mFcγRIV; horse IgG complexes only to mFcγRI above background levels observed with untransfected CHO cells (Figures 1N, S1I). Cow IgG and chicken IgY complexes did not bind. Importantly, we observed a high unspecific binding of the Fab2-anti cow secondary antibody to all CHO lines, possibly due to cell culture in presence of fetal bovine serum. This unspecific binding could mask weak interactions of cow IgG complexes with mFcγRIII (and mFcγRIV) that seem to present a higher fluorescent signal, when compared to untransfected CHO cells.

IgG binding to human FcγRs

To assess the binding of IgGs from different species to human FcγRs and their polymorphic variants, hFcγRI, hFcγRIIA_(H131 and R131), hFcγRIIB, hFcγRIIIA_(F158 and V158), and hFcγRIIIB_(NA1, NA2, and SH), we used a collection of CHO cells transfected with FLAG-tagged hFcγRs sorted to express comparable levels of each hFcγR[2] (Figures 2A, S2A).

Complexes containing *human* IgG1, IgG3 and IgG4 bound to all hFcγRs, but with different overall binding strength: IgG3>IgG1>IgG4 (Figures 2C/H, S2B). Human IgG2 ICs showed binding restricted to hFcγRIIA, hFcγRIIB and hFcγRIIIA_{V158}. These datasets are largely in agreement with our study published in 2009 using polyclonal human IgG subclasses, but reveal additional interactions: IgG2 interactions with hFcγRIIB [2, 27], and IgG4_{S228P} interactions with hFcγRIIIA_{F158}[27] and the three hFcγRIIIB variants. We confirm that hFcγRIIB has the overall weakest capacity to bind IgG

among all hFcγRs, and that hFcγRIIIA_{V158} shows a higher avidity for IgG complexes than its polymorphic variant hFcγRIIIA_{F158}[2, 5].

Macaque IgG complexes showed very broad binding to hFcγR, as reported previously[11], with weaker interactions becoming undetectable using Fab₂Cs (Figures 2D/I, S2C).

Mouse IgG complexes demonstrated few interactions with hFcγRs (Figures 2E/J, S2D), as anticipated[13]. Mouse IgG1 and IgG2b complexes bound predominantly to hFcγRIIA_{R131} and hFcγRIIA_{H131} and weakly to hFcγRI and hFcγRIIB; mouse IgG2a and IgG2c complexes to all hFcγRs, with the exception of hFcγRIIB variants; and IgG3 complexes to FcγRI and weakly to hFcγRIIIA_{V158}.

Rabbit ICs bound to all hFcγRs (Figures 2F/K, S2E), which is in agreement with the proven ability of rabbit IgG to trigger strong activation of human neutrophils *in vitro*[28].

Fab₂Cs made of *rat* IgG demonstrated subclass-specific binding to hFcγRs (Figures 2L, S3B). Rat IgG1 Fab₂Cs bound predominantly to hFcγRIIA_{R131}; rat IgG2a complexes did not show significant binding, a feebly interaction with hFcγRIIA_{R131} was detected; rat IgG2b complexes bound with variable avidity to all hFcγR with the exception of hFcγRIIB.

Total *hamster* IgG (S/A) and IgG1 (A) complexes bound essentially to hFcγRIIA_{R131} with some weak detectable binding of Armenian hamster bulk IgG and IgG1 complexes to hFcγRIIA_{H131}. Armenian hamster “IgG3” failed to bind significantly to any hFcγR (Figures 2M, S3C).

Other: Unexpectedly, guinea pig IgG complexes demonstrated strong and selective interaction with hFcγRI, hFcγRIIIA and hFcγRIIB variants (Figures 2N, S3D). Complexes made of goat IgG weakly interacted with hFcγRIIA_{R131}, hFcγRIIIA_{V158} and hFcγRIIB, as reported[29]. A similar pattern was observed for sheep IgG complexes, albeit with weaker intensities. Again, the high background fluorescence of the anti-cow F(ab')₂ to CHO cells, precluded reliable detection of cow IgG complex binding to hFcγRs. The shifts in fluorescence however suggest a similar binding profile as sheep and goat IgG. No significant binding with hFcγRs could be observed for either horse IgG or chicken IgY Fab₂Cs.

In conclusion, it is to be expected that IgG from species other than humans and mice will interact with at least one, and mostly a sizeable fraction of, human and mouse Fc γ Rs. A few atypical IgG make the exception, but this can be explained either by the low overall similarity between their CH2/CH3 domains and those of human or mouse IgG, (example: rat IgG2a, Figure 3) or within the regions making the contact to the Fc γ R (example: chicken IgY, Figure S4). Close phylogenetic relationships of IgG from different species generally translate into similar binding patterns to Fc γ Rs. As expected[5], large ICs demonstrated more extended binding than the smaller Fab₂Cs. Our study presents some limitations: We used transfectants expressing high levels of Fc γ R, whose density may allow interactions that do not occur or are insufficient to engage Fc γ Rs *in vivo*. Furthermore, although we aimed at using F(ab')₂-fragment specific IgG, whenever possible (human, mouse, rat, rabbit, goat), the Fab₂Cs from other species were generated using anti-IgG (H+L) F(ab')₂ fragments (Table S1) that may alter the binding of the IgG Fc domain to Fc γ Rs.

Collectively, our data draw a comprehensive map of interactions between IgG from various species and mouse and human Fc γ Rs. It allows inferring Fc γ R effector functions triggered by each of these IgG subclasses for *in vivo* and cell-based *in vitro* experimentation and highlights the necessity to consider Fc-mediated interactions in antibody-based immunoassays.

Materials and Methods

Cells

Stably transfected CHO-K1 expressing either human or mouse FLAG-tagged Fc γ Rs were cultured as described[2],[15]. Cells were used for binding experiments 3 days after passage. Ig binding, transfection level and Fc γ R expressed (Figure 1A/2A) were analyzed by flow cytometry (MACSQuant10/16, Miltenyi Biotec)[30]. The full gating strategy is depicted in Supplemental Figure 5.

Antibodies and reagents

Bovine serum albumin (BSA) (Sigma-Aldrich) was tri-nitrophenylated by incubation with picric acid (Eastman Kodak) and the product fractionated on a gel filtration column (AKTA, GE Healthcare). Collected TNP₃₃-BSA was biotinylated using the Pierce Biotin-Conjugated Molecule kit (Thermo Fisher). The hybridoma producing mAbs mouse IgG2a anti-TNP (Hy1.2) were provided by S. Izui (University of Geneva, Geneva, Switzerland), IgG2b anti-TNP (GORK) by B. Heyman (Uppsala Universitet, Uppsala, Sweden) and IgG3 anti-TNP (C3110E3) by J. Van Snick (Ludwig Institute for Cancer Research Ltd, Brussels, Belgium). Codon-usage optimized variable regions of the mouse H and L chain hybridoma IGELa2 (X65772.1, X65774.1) were cloned into human pUC19-Ig γ 1-or -Ig κ expression vectors (a kind gift from Hugo Mouquet, Institut Pasteur, Paris), respectively[31]. Antibody switch variants were generated by replacement of the human L or H chain C regions by human C regions with IgG2, IgG3 and IgG4_{S228P}; mouse κ L chain (AJ487682.1) or γ 2b H chain C regions (J00479.1); Cynomolgus κ L chain (JN984930) or γ 1-4 H chain C regions (IgG1: JN984927; IgG2: JN984928; putative IgG3: DJ444798, IgG4: JN984929[11] – gene synthesis: Synbio Technologies). All in house produced antibodies were obtained by FectoPRO (Polyplus) transfection of Expi cells purified on a ProteinG column followed by a desalting column on an HPLC instrument (AKTA, GE Healthcare).

Immunoglobulin binding assays

IgG complexes were formed as: *i*) *F(ab')₂-aggregated IgG complexes*, pre-formed by incubating 10 μ g/mL IgG with 5 μ g/mL fluorescently-labeled anti-IgG F(ab')₂ fragments (Supplemental Table 1) in PBS 0.05% BSA 2 μ M EDTA, pH7.4 for 30 minutes at 37°C; or *ii*) *immune complexes (ICs)* made of 10 μ g/mL anti-TNP IgG mAbs incubated with 3.3 μ g/mL TNP₃₃-BSA-biotin for 30 min at 37°C. 2×10^5 CHO cells expressing human or mouse FLAG-tagged Fc γ Rs[2],[15] were incubated with either of

these types of IgG complexes for 30 minutes at 4°C and washed. Cell-bound TNP ICs were revealed using 1 µg/mL APC-labeled streptavidin, heat aggregates by incubating cells with 5 µg/mL fluorescently labeled anti-IgG F(ab')₂ fragments for 30 minutes at 4°C prior to acquisition. Binding is reported as ΔMFI of IgG complex binding–control (streptavidin or fluorescently-labeled anti-IgG F(ab')₂ fragments).

Phylogenetic analysis

IgG CH2 and CH3 protein sequences were extracted from sequences listed in Supplemental Figure 4 according to IMGT[32] annotations and aligned using MUSCLE software[33, 34] with the exception of chicken IgY. The obtained alignment was submitted to phylogenetic analysis using PhyML 3.0[35] with default parameters. The phylogenetic tree was visualized using iTOL[36], branches with a bootstrap of <0.5 were deleted, colors indicate major clades.

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Authorship Contributions

YW and VK performed binding experiments. BI, OR, LR, DAM, PB and FJ prepared cell lines and reagents. FJ and LdC supervised the work, YW, PB and FJ wrote the manuscript.

Disclosure of Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Legends

Figure 1: Summary of the binding of IgG/Y complexes from indicated species to mFcγRs.

(A) Histograms depict fluorescent intensity of FLAG-tagged mFcγR CHO transfectants and stained or not (grey) with anti-Flag (red) or anti-FcγR mAbs (green). Heatmaps summarizing log₁₀ transformed MFI of control stainings (anti-Flag, unstained, Streptavidin control) (B/G) and binding of preformed complexes of mouse (C/H), human (D/I), macaque (E/J), rabbit (F/K), rat (L), hamster (M), and guinea pig, cow, sheep, goat, horse IgG and chicken IgY (“others” (N)) to FLAG-tagged mFcγRs on CHO transfectants. Binding of preformed biotinylated TNP ICs (C-F) was revealed by Streptavidin-APC and Fab₂Cs (H-N) were formed by aggregating IgG with fluorescently labeled-F(ab')₂ anti-IgG (F(ab')₂ fragment-specific for anti-mouse, human, rabbit, rat and goat). Data represent means of at least three independent experiments.

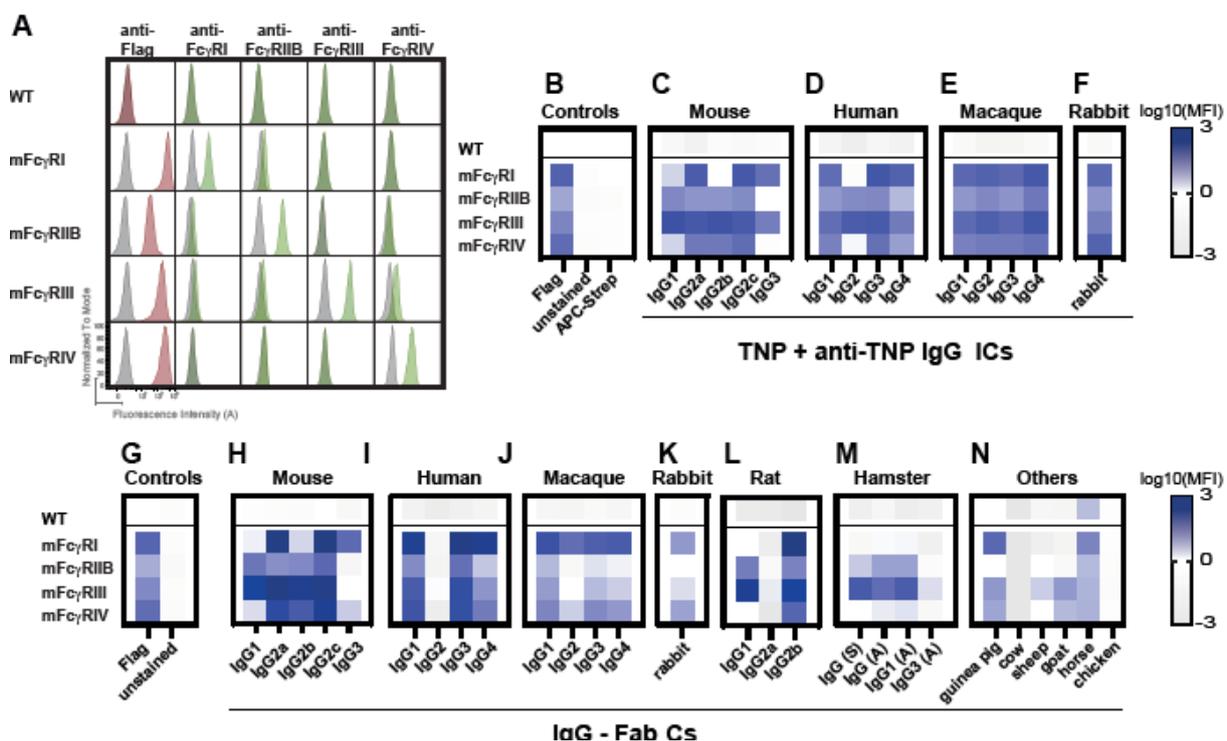


Figure 2: Binding specificity of hFc γ R for IgG/Y complexes from indicated species.

(A) Histograms depict fluorescent intensity of FLAG-tagged hFc γ R CHO transfectants and stained or not (grey) with anti-Flag (red) or anti-Fc γ R mAbs (green). Heatmaps summarize log₁₀ transformed MFI of control stainings (anti-Flag, unstained, Streptavidin control) (B/G) and binding of preformed complexes of human (C/H), macaque (D/I), mouse (E/J), rabbit (F/K), rat (L), hamster (M), and guinea pig, cow, sheep, goat, horse IgG and chicken IgY (“others” (N)) as ICs (C-F) or Fab₂Cs (H-N) to the cells. Data are representative of at least 3 independent experiments.

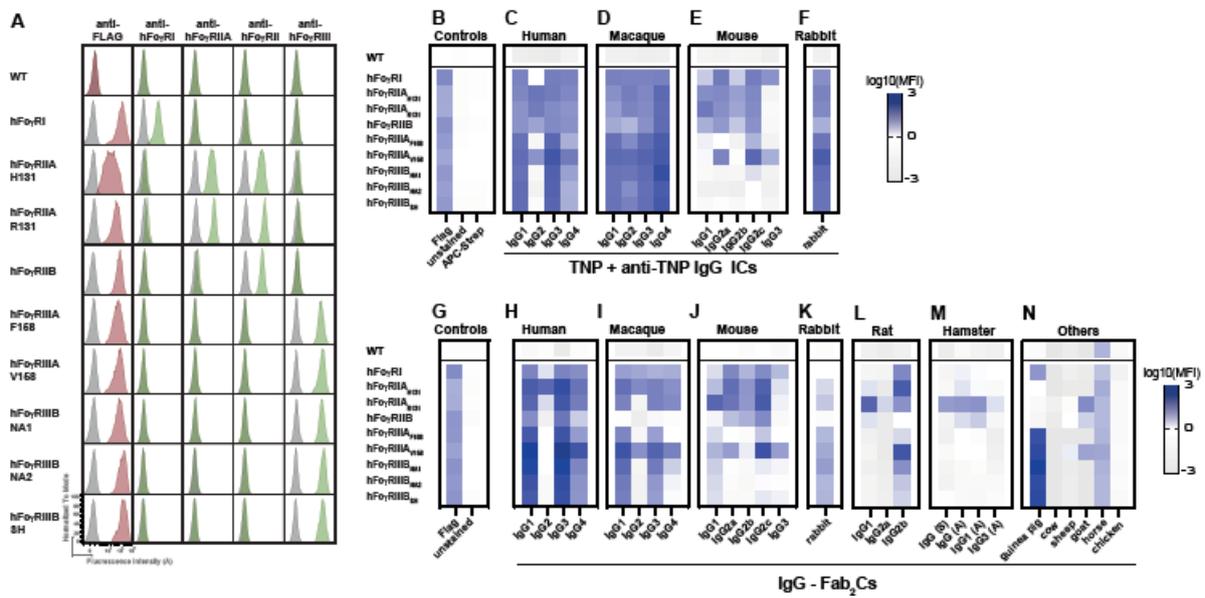
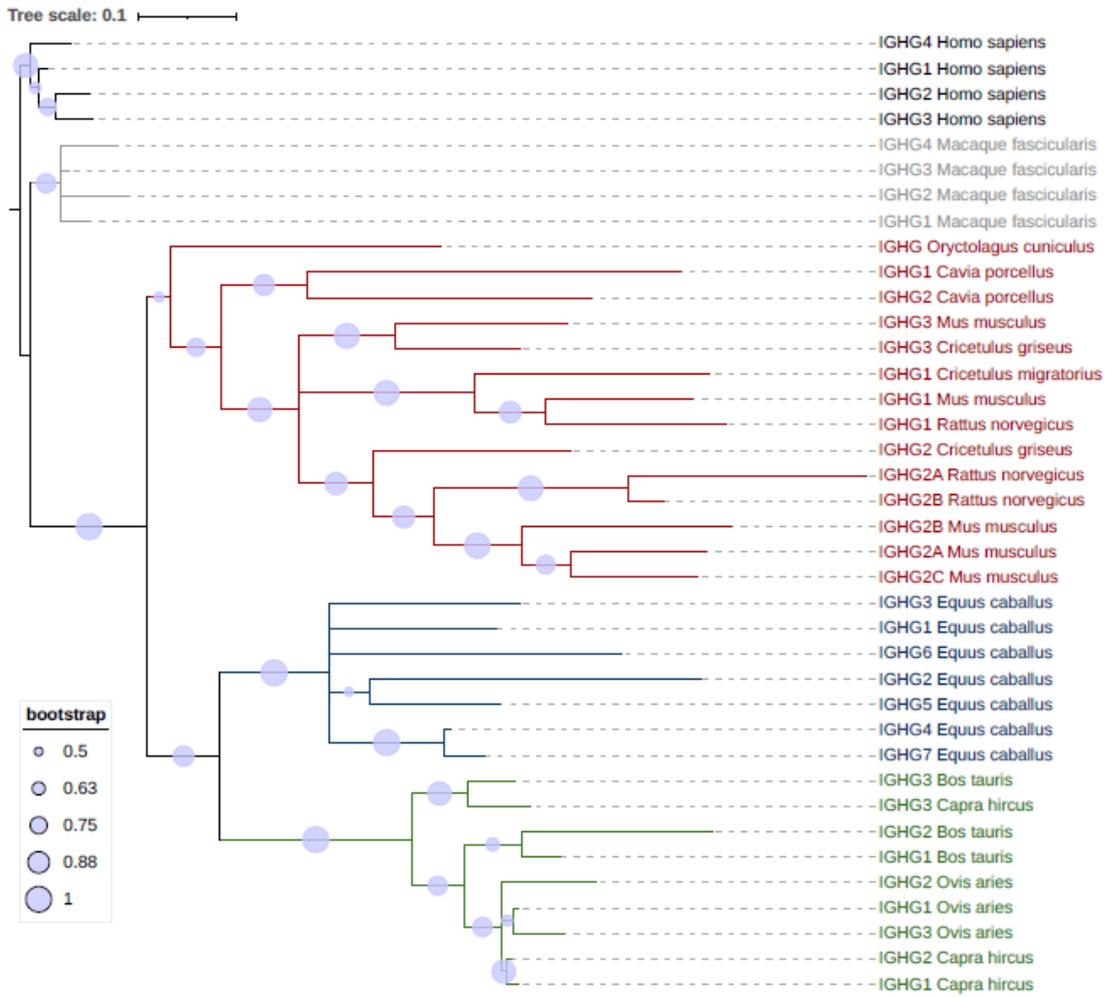


Figure 3: Phylogenetic tree of IgG CH2-CH3 domains of IgG.

Phylogram of IgG CH2 and CH3 protein sequences (Supplemental Figure 5 excluding chicken), branches with a bootstrap of <0.5 were deleted, colors indicate major clades.



We undertook a systematic assessment of binding specificities of IgG from various species to mouse and human FcγRs, including their polymorphic variants. Our data draw a comprehensive map of their interactions and reveal that most IgG interact with at least one, and mostly several, human and mouse FcγRs.

