Cross-species higher sensitivities of FcγRIIIA/FcγRIV to afucosylated IgG for enhanced ADCC

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Received: June 30, 2021; Revised: July 26, 2021; Accepted: August 13, 2021

Abstract

Background: Expressing afucosylated human IgG1 antibodies with Chinese hamster ovary (CHO) cells deficient of α-(1,6)-fucosyltransferase (FUT8) is being more and more accepted as a routine method to enhance antibody-dependent cellular cytotoxicity (ADCC) of therapeutic antibodies, especially for anti-cancer regimens. However, in pre-clinical studies relying on disease models other than mice and primates, e.g., those underrepresented species for infectious diseases, it is less clear whether such afucosylated antibodies can demonstrate enhanced therapeutic index. This is because the orthologues of human FcγRIIIA or mouse FcγRIV from those species have not been well characterized.

Methods: We set up a luciferase-based ADCC assay with Jurkat reporter cells expressing FcγRIIIA/FcγRIV from human, mouse, rat, hamster, guinea pig, ferret, rabbit, cat, dog, pig and monkey, and also produced human, mouse, hamster, rabbit and pig IgG from wild type and Fut8−/− CHO cells or hybridomas.

Results: We confirmed that enhanced stimulation through FcγRIIIA/FcγRIV by afucosylated IgG, as compared with wild type IgG, is a cross-species phenomenon.

Conclusions: Thus, efficacy and toxicology studies of the next generation afucosylated therapeutic IgG and Fc fusion proteins in these underrepresented animal models should be expected to generate translatable data for treating human diseases, leading to the expanded applications of this new class of glycoengineered biologics.

Statement of Significance: Orthologues of hFcγRIIIA from mouse, rat, hamster, guinea pig, ferret, rabbit, cat, dog, pig and monkey exhibit higher sensitivities towards afucosylated IgG for enhanced ADCC, facilitating efficacy, toxicity and MOA studies of the next generation afucosylated therapeutic IgG and Fc fusion proteins directly in underrepresented animal models of human diseases.

KEYWORDS: IgG; afucosylation; FcγR; ADCC; cross-species

INTRODUCTION

Antibodies through their fragment crystallizable (Fc) region engagement of Fc receptors can exert potent antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) activities towards cancerous and/or infected cells. The major mechanisms of action (MOA) for most therapeutic IgG antibodies in cancer treatment, e.g., are via ADCC and ADCP. To enhance ADCC/ADCP, one approach is to introduce mutations in IgG Fc region to increase its binding towards human FcγRIIIA [1–3]. However, this may also create antigenic epitopes and trigger anti-drug antibodies. Another approach is through glycoengineering to generate afucosylated IgG antibodies [4, 5]. The Asn297-linked N-glycans in human IgG1 Fc are...
bi-antennary complex-type composed of a tri-mannosyl core structure with or without core fucose residues. Afucosylated forms of human IgG1 are observed as natural variants (~10%) in normal human serum IgGs, hence are not immunogenic. Afucosylated human IgG1 exhibit dramatically increased ADCC due to the enhancement of FcγRIIIA binding capacity without any detectable change in complement-dependent cytotoxicity or antigen binding capability [6–9]. Recent findings indicate that the lack of core fucose also promotes ADCP mediated by FcγRIIA-positive monocytes and macrophages, especially under conditions that more closely resemble the physiologic settings when the competing endogenous serum IgG is present [10, 11].

Although it has been strongly proposed that the next generation anti-cancer IgGs should all be afucosylated if their MOAs involve ADCC/ADCP [12], there are conditions that afucosylated IgGs can cause pathogenic consequences. In certain autoimmune disease, e.g., fetal or neonatal alloimmune thrombocytopenia, the triggering pathological IgGs are afucosylated [13]. In dengue and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections, afucosylated IgG1 appear to induce excessive immune-mediated damage to patients, and the levels of afucosylated IgG1 are predictive of dengue and coronavirus disease 2019 (COVID-19) disease severity [14–17]. On the other hand, afucosylated IgG antibodies confer more potent immunoprotection against Ebola virus [18, 19], very likely through more efficient clearing of virus-infected cells. Although such antibodies increase ADCC towards HIV-infected cells in vitro [20], improved protection against mucosal simian-human immunodeficiency virus (SHIV) challenge in macaques was not observed [21]. Thus, especially in infectious diseases, whether afucosylated IgGs have better therapeutic index highly depends on the mechanisms of disease pathogenesis and the MOA of the treating antibodies.

Another crucial factor restraining the application of afucosylated antibodies is the limited knowledge of cross-species translatability of the observed effects. In infectious disease research, often times scientists have to rely on certain underrepresented animal models. For example, ferrets are a well-established model [22, 23] for evaluating antiviral therapies and studying the pathogenesis and transmission of human respiratory viruses, including influenza and SARS-CoV-2 [24, 25]. Although viral replication of SARS-CoV-2 can be studied in ferrets, fatality was not observed [24]. Hamsters have also been used to study SARS-CoV-2 [26, 27]. The Roborovski dwarf hamster (Phodopus roborovskii) is a highly susceptible COVID-19 model with consistent and fulminating clinical signs. Especially, only this species shows SARS-CoV-2-induced severe acute diffuse alveolar damage and hyaline microthrombi in the lungs, changes described in patients who succumbed to the infection but not reproduced in any other experimentally infected animals [28]. When testing recombinant human IgGs or mouse hybridoma antibodies in these underrepresented disease models, it becomes imperative to understand the cross-species interactions between therapeutic antibodies and species-specific FcγRs, particularly, whether afucosylated IgGs have similar enhancement in ADCC/ADCP observed in the human system.

When examining the homologues of human FcγRIIIA in various animal species, we noticed that there is ample sequence information derived from automatic bioinformatics annotation, but scarce functional data are available on this low affinity immunoglobulin gamma Fc receptor from underrepresented animal species. Some homologues are mistakenly annotated as different family members, whereas others are not even characterized and curated. This status quo severely hampered the application of afucosylated IgGs as the next generation therapeutics. In 2016, we identified a previously uncharacterized protein (H0VDZ8 from UNIPROT database) as the guinea pig (Cavia porcellus) FcγRI that exhibits enhanced binding to afucosylated human and mouse IgG [29]. This crucial information directly supported the testing of afucosylated MBP134AF, a pan-ebolavirus human antibody cocktail, in the guinea pig infection model, and found that MBP134AF is more efficacious than the wild type version [19].

After systematic construction of a panel of species-specific FcγRIIIA/FcγRI reporter cell lines and side-by-side expression of multiple pairs of wild type (WT) and afucosylated (AF) IgG antibodies (targeting h5T4, hMUC-1, mCD39 and mCD79b), using WT Chinese hamster ovary (CHO)-K1 or our glycoengineered Fut8−/− CHO-K1 cell line, as well as WT or Fut8−/− hybridomas, we now report that enhanced stimulation through FcγRIIIA/FcγRIV by afucosylated IgG, as compared with WT IgG, is a cross-species phenomenon. Thus, IgG afucosylation by using Fut8−/− production cell lines can act as an effector function amplifier in a wide range of animal species. This simple strategy can turn some otherwise inactive antibodies into highly effective ones in certain species. Our findings provide the foundation for rational pre-clinical efficacy and toxicology studies of afucosylated therapeutic antibodies directly in various animal models of human diseases.

**MATERIALS AND METHODS**

**Expression of WT and afucosylated antibodies**

The heavy and light chain genes of anti-h5T4-hIgG1, anti-hMUC1-C-hIgG1, anti-mCD79b-hmstr-IgG2, as well as chimeric anti-mCD79b-hmstr-IgG1, anti-mCD79b-rbt-IgG and anti-mCD79b-pig-IgG constructed by overlapping polymerase chain reaction (PCR) were all cloned into pDirect7.0 CHO expression vector (Antagen, MA), sequenced verified (Genewiz, NJ) and then electroporated (1620 V, pulse width 10 ms, 3 pulses) with the Neon™ electroporation system (LifeTech, CA) into WT or Fut8−/− CHO-K1 cells (Antagen). One day after electroporation, cells were selected with Zeocin (400 μg/mL) in DMEM-5%FBS for 2 weeks. Drug-resistant colonies were pooled together and transferred to HyCell serum-free medium (Global Life Sciences Solutions, MA) in shaking culture for 2–3 weeks. Culture supernatants were filtered and loaded into Protein A columns to purify the antibodies.

For hybridoma-derived anti-mCD39 antibodies, 96-well subcloning was performed to obtain clonal lines with the original mIgG1 kappa isotype and the spontaneously
class-switched mIgG2c kappa isotype, screened and confirmed by ISO-M8c rapid mouse immunoglobulin isotyping XpressCard™ (Antagen). To generate the Fut8− hybridomas, oligos for guide RNAs targeting the murine Fut8 gene were cloned into sgRNA/Cas9n expression vector pX335 (Addgene, MA) as previously described [30], and electroporated into WT anti-mCD39-mIgG1 and anti-mCD39-mIgG2c cells. After electroporation, cells were cultured in DMEM-10%FBS for 8 days before stained with biotinylated Lens Culinaris Agglutinin (LCA; Vector Laboratory, CA), followed by Streptavidin-PE (eBiosciences, CA) and anti-PE-microbeads (Miltenyi Biotec, CA). The cell suspensions were loaded onto MACS® columns and the pass-through cells were subcloned in 96-wells to obtain clonal lines. Flow cytometry was used to confirm positive binding to the WT and negative binding to Fut8− hybridoma lines by LCA lectin. All the hybridoma lines were cultured in DMEM-10%FBS, and supernatants were loaded onto Protein L columns to purify the antibodies.

Stable CHO cell lines expressing surface antigens as ADCC target cells

Stable CHO cell lines expressing mouse CD39, mouse CD79b and human ST4, respectively, were generated with the “Toggle-In” system (Antagen). The mCD39 (UNIPROT: P55772) and mCD79b (UNIPROT: P15530) open reading frames (ORFs) were PCR cloned from mouse spleenocyte cDNA library, and the hST4 (UNIPROT: Q13561) ORF was fully synthesized (Twist Biosciences, CA). All the genes were cloned into pTOG3 vector (Antagen) and the inserts were sequencing confirmed (Genewiz). 1.0 μg of each pTOG3 construct was co-transfected with 20 ng Cre-encoding pOG231 plasmid (Addgene) into CHO-E1 cells (Antagen) at a transcriptional “hot-spot” via Cre-LoxP recombination-mediated cassette exchange, followed by Hygromycin B selection (800 μg/mL) for 10 days. Single CHO clones were picked and confirmed by reverse transcription polymerase chain reaction (RT-PCR) and Fluorescence-activated cell sorting (FACS) staining with antibodies. All the clones within each line were isogenic with the same genomic integration by the “Toggle-In” method. An HCT116 cell line overexpressing human MUC-1-C was provided by Genus Oncology, limited liability corporation (LLC). Clonal lines were used as target cells in ADCC assays.

Jurkat reporter cell lines expressing species-specific FcγR as ADCC effector cells

All the species-specific FcγR genes were fully synthesized (Twist Biosciences, CA) and cloned into the lentiviral vector pHAGE2-FullEFlα-ZsGreen-IRESDTOMato-W-T, which harbors an insert of TdTomato as an infection indicator, followed by lentivirus packaging in HEK293 cells as previously described [31]. Jurkat T cells (ATCC, VA) were co-transfected with pGL4.30 luc2P/NFAT-RE/Hygro vector (Promega, WI) with the luciferase reporter under the control of the NFAT promoter, as well as Murine Maloney Leukemia Virus-based vector pLNCX2 (Takara Bio, CA) expressing human Fc gamma common chain with a neomycin resistance cassette. The Hygromycin B and G418 dual-resistant Jurkat cells were further transduced with lentivirus expressing species-specific FcγR, and TdTTomato+ cells were FACS-sorted and maintained in RPMI-10%FBS with 200 μg/mL G418 and Hygromycin B (In VivoGen, CA).

RESULTS

Characterization and phylogenetic comparison of FcγRIIa/FcγRIV from different mammalian species

The murine orthologue of human FcγRIIa (CD16a) is mFcγRIV. Its expression is restricted to myeloid lineage cells, and binds to mIgG2a and mIgG2b with intermediate affinity, but no binding to mIgG1 or mIgG3 was observed [32]. Experiments with FcγR knockout mice showed that mFcγRIV, but not mFcγRI or mFcγRIII, plays a central role in mIgG2a/b/c mediated tumor rejection [33]. We used mFcγRIV to search the UNIPROT database, and found a highly homologous protein (H0VDZ8) from guinea pig (C. porcellus). Similar to hFcγRIIa and mFcγRIV, H0VDZ8 demonstrated higher binding to afucosylated hIgG1 (Supplementary Fig. S1) and mIgG2a than their WT counterparts, hence we named it as guinea pig FcγRIV (gpFcγRIV) [29].

Using gpFcγRIV as bait, we blasted the UNIPROT database and found an uncharacterized protein (M3XWH1) from European domestic ferret (Mustela putorius furo) that shows high homology to gpFcγRIV. The protein database of the National Center for Biotechnology Information (NCBI) annotates this very same sequence (XP_004751441) as ferret FcγRIII-like. An almost identical protein (M1EQN2) in the UNIPROT database with only two amino acids (aa) missing at the very end of M3XWH1 is annotated as low affinity FcγRIIIa.

In a similar attempt, we blasted gpFcγRIV against the UNIPROT database and found two uncharacterized proteins (G1T7E7 and G1TR84) from rabbit (Oryctolagus cuniculus) that are highly homologous to gpFcγRIV. G1T7E7 and G1TR84 are located in different regions on chromosome 13 with only 4 nt different at the DNA level, resulting in 4 aa disparate out of total 253 aa, i.e., most likely they were derived from gene conversion events. The NCBI protein database annotates both (XP_002715295,
Figure 1. Comparison of the amino acid sequences of FcγRIIIA or FcγRIV from different mammalian species. (A) Alignment of the full-length sequences of FcγRIIIA or FcγRIV with ClustalW via the server at http://www.phylogeny.fr. Similar residues are colored in cyan as the most conserved ones (according to BLOSUM62 score). Average BLOSUM62 score: Max: 3.0 (cyan), Low: 0.5 (grey). YEEP motifs in the intracellular region of rodent FcγRIV are boxed in red. (B) Phylogenetic tree of the relationships among members of the greater FcγRIIIA/FcγRIV family. The tree topology was estimated with “neighbor joining” in which branch values (numbers above lines) represent percent “bootstrap” support (values below 50% are not included). Scale bar, genetic distance. Amino acid sequences were derived from the following (UNIPROT or NCBI identifiers in parentheses): mouse FcγRIII (P08508), mouse FcγRIV (A0A0B4J1G0), rat FcγRIII (P27645), rat FcγRIV (A0A0B4J2J1), guinea pig FcγRIII (H0V371), guinea pig FcγRIV (H0VDZ8), golden hamster FcγRIII (A0A3Q0CHI5), golden hamster FcγRIV (A0A1U7Q211), Chinese hamster FcγRIII (XP_007645600), Chinese hamster FcγRIV (XP_035301700), ferret FcγRIIIA (M3XWH1), rabbit FcγRIIIA (G1TR84), rabbit FcγRIIIA (G1T7E7), cat FcγRIIIA (Q9N2I5), dog FcγRIIIA (E2RP87), pig FcγRIIIA (Q28942), monkey FcγRIIIB-like (XP_00715293) as rabbit low affinity immunoglobulin gamma Fc region receptor III-B-like.

We compared the amino acid sequences of FcγRIII and FcγRIV from various mammals using BLAST search results from the UNIPROT and NCBI databases (Fig. 1A). Phylogenetic comparison of the full-length amino acid sequences for this collection of mammalian FcγRIII and FcγRIV receptors indicated that rodent FcγRIV receptors are more closely related to non-rodent FcγRIIIA orthologues than they are to the rodent FcγRIII receptors (Fig. 1B). Interestingly, the phylogenetic relationships of rodent FcγRIV receptors perfectly mirror those of rodent FcγRIII receptors, i.e., the two hamster genes are as close as the mouse and rat genes, but the four only remotely resemble the guinea pig gene (Supplementary Fig. S2). This suggests that rodent FcγRIV genes separated from rodent FcγRIII genes at an early time point during evolution, supporting that they have distinct functions.
Afucosylated mouse IgG2c but not IgG1 exhibits enhanced ADCC via FcγRIV orthologues

Given the drastic difference in effector functions of the activating murine FcγRIV vs. FcγRIII receptors [33], it is important to functionally characterize the FcγRIIIA/FcγRIV receptors from different mammalian species, and give them the right nomenclature to guide \textit{in vivo} studies. Because mouse FcγRIV only binds to mIgG2a/b/c isotypes, whereas mouse FcγRIII also binds to mIgG1 in addition [32, 35], we used a panel of WT and afucosylated (AF) mIgG2c and mIgG1 to perform the ADCC assay. Note that, mouse IgG2a and IgG2c genes are not linked but allelic [36]. Although BALB/c mice express IgG2a, some other inbred mouse strains such as C57BL/6, C57BL/10 and NOD do not have the gene for IgG2a but instead express the IgG2c isotype [36].

We determined that a mouse hybridoma (5F2) of C57BL/6 background against mouse CD39 is of mIgG1 isotype, and also cloned its spontaneously class-switched mIgG2c subtype derived from a rare event during a culture crash incident. DNA sequencing of the RT-PCR-amplified heavy chain genes confirmed that this was an authentic class-switching event (not shown). The Fut8$^{-/-}$ 5F2 mIgG1 and mIgG2c sublines were subsequently established after CRISPR-mediated knocking out of the murine Fut8 gene, and confirmed by negative staining with fucose-binding LCA (not shown). Thus, we have set up a perfect panel of four mouse hybridoma antibodies with the identical Fab portion for the same antigen recognition, but with mIgG1 vs. mIgG2c isotypes and WT vs. AF glycan profiles.

In ADCC assays using mCD39-expressing CHO cells as target cells and luciferase-equipped Jurkat cells expressing mFcγRIV as effector cells, AF mIgG2c is 50–100-fold more potent than WT mIgG2c in mediating ADCC, whereas mIgG1 antibodies, regardless of WT or AF, are completely ineffective (Fig. 2A). In the same system but with Jurkat reporter cells expressing mFcγRIII, AF mIgG2c also exhibits enhanced ADCC compared with WT, although their EC$_{50}$ concentrations are ∼10-fold higher than those with mFcγRIV (Fig. 2B). Interestingly, mIgG1 stimulates mFcγRIII for ADCC at even lower concentrations, but mFcγRIII does not differentiate the content of fucose on mIgG1, and the two ADCC curves of WT and AF mIgG1 are completely overlapped (Fig. 2B). These results are in line with the reports that mFcγRIII but not mFcγRIV also binds to mIgG1 [32, 35]. Thus, our 5F2 mIgG1/mIgG2c combo set and ADCC assay can functionally differentiate whether a rodent FcγR homologue should be classified as FcγRIII or FcγRIV, i.e., a receptor that is hyperstimulated by the afucosylated form of mIgG2c but not by mIgG1 should be an FcγRIV orthologue. Conversely, a receptor that cannot differentiate the WT vs. AF forms of mIgG1 should be an FcγRIII orthologue.

With this in mind, we tested 5F2 mIgG1/mIgG2c combo set in ADCC assays with luciferase-equipped Jurkat cells expressing species-specific FcγRIII/FcγRIV. The patterns of ADCC response are overall very similar, with a few exceptions, among FcγRIV receptors from mouse, rat, hamster, guinea pig, and FcγRIIIA receptors from ferret, rabbit, cat, dog, pig, monkey and human (Fig. 2C–N). That is, afucosylation of mIgG2c enhances ADCC over WT mIgG2c for at least 10-fold (in pig and guinea pig) and usually >20–100-fold in most other mammalian species. Not only the effective concentrations of AF mIgG2c antibody are greatly reduced, but also the magnitudes of ADCC response are dramatically increased. For golden hamster FcγRIV, rabbit and cat FcγRIIIA, simple afucosylation transforms an otherwise ineffective WT mIgG2c into a highly effective antibody with potent ADCC (Fig. 2E, H–J). On the contrary, AF mIgG1 does not trigger any ADCC response by FcγRIV from mouse and hamster, or by FcγRIIIA from ferret, rabbit (G1T7E7), cat, dog, monkey and human (Fig. 2A, D, E, G, I–K, M, N), confirming that they are all orthologues of the murine FcγR. Interestingly, perhaps due to cross-species binding, gpFcγRIV and pig FcγRIIIA do respond to WT mIgG1, and the responses are further enhanced by afucosylation of mIgG1 (Fig. 2F, L). Similar enhanced responses to AF mIgG1 are also seen with rat FcγRIV and rabbit FcγRIIIA (G1TR84), although at much lower levels (Fig. 2C, H). Clearly, these FcγRs are different from mFcγRIII in response to AF mIgG1 (Fig. 2B).

Afucosylated hamster IgG2 but not IgG1 also exhibits enhanced ADCC via FcγRIIIA/FcγRIV orthologues

To rule out the possibilities that the observed effects were specific for mouse hybridoma-derived antibodies or for the recognized mCD39 antigen only, we used WT and Fut8$^{-/-}$ CHO cells to recombinantly express an Armenian hamster (\textit{Cricetus miliarius}) anti-mouse CD79b antibody (clone HM79-16 or HM79b; [37]). The original HM79–16 hamster antibody has IgG2 heavy chain (GenBank: AGH06133) and lambda light chain (GenBank: AGH06134). We also generated a hamster IgG1 version of HM79-16 by replacing its Fab domain with the Fab of Armenian hamster IgG1 antibody HL4E10 (GenBank: AEG80191). Alignment analyses indicate that hamster IgG2 (hmstr-IgG2) and hmstr-IgG1 have high homologies with mouse IgG2a and mIgG1, respectively (not shown). Similarly, we engineered two additional chimeric HM79-16 antibodies with the same original Fab in fusion with FcγRIIIA (Fig. 2B), many mammalian species. Not only the effective concentrations of AF mIgG2c antibody are greatly reduced, but also the magnitudes of ADCC response are dramatically increased. For golden hamster FcγRIV, rabbit and cat FcγRIIIA, simple afucosylation transforms an otherwise ineffective WT mIgG2c into a highly effective antibody with potent ADCC (Fig. 2E, H–J). On the contrary, AF mIgG1 does not trigger any ADCC response by FcγRIV from mouse and hamster, or by FcγRIIIA from ferret, rabbit (G1T7E7), cat, dog, monkey and human (Fig. 2A, D, E, G, I–K, M, N), confirming that they are all orthologues of the murine FcγR. Interestingly, perhaps due to cross-species binding, gpFcγRIV and pig FcγRIIIA do respond to WT mIgG1, and the responses are further enhanced by afucosylation of mIgG1 (Fig. 2F, L). Similar enhanced responses to AF mIgG1 are also seen with rat FcγRIV and rabbit FcγRIIIA (G1TR84), although at much lower levels (Fig. 2C, H). Clearly, these FcγRs are different from mFcγRIII in response to AF mIgG1 (Fig. 2B).
Figure 2. Afucosylated mouse IgG2c but not IgG1 exhibits enhanced ADCC via FcγRIV orthologues. The original mouse anti-mCD39 hybridoma (clone 5F2, mIgG1) was screened to select a spontaneously class-switched subline with mIgG2c isotype. Both clonal 5F2-mIgG1 and 5F2-mIgG2c hybridomas were rendered Fut8−/− by CRISPR technology to produce afucosylated (AF) antibodies. Purified WT and AF 5F2 antibodies with different isotypes and fucose content all have the exact same Fab, recognizing mCD39 with equal strength (not shown), but demonstrated drastic differences in ADCC assays with various Fcγ receptors. Compared with WT 5F2-mIgG2c, AF 5F2-mIgG2c exhibits greatly enhanced ADCC via mouse FcγRIV (A), mouse FcγRIII (B), rat FcγRIV (C), Chinese hamster FcγRIV (D), golden hamster FcγRIV (E), guinea pig FcγRIV (F), ferret FcγRIIA (G), rabbit FcγRIIA (G1TR84) (H), rabbit FcγRIIA (G1T7E7) (I), cat FcγRIIA (J), dog FcγRIIA (K), pig FcγRIIA (L), monkey FcγRIIA (M) and human FcγRIIA (N). Note that mouse FcγRIII mediates equal ADCC responses regardless of the fucose content on mIgG1 (B), a feature that is not seen among all the other FcγIV orthologues. Data are summarized from at least two independent experiments with Mean ± standard deviation (SD) calculated from repeat experiments when luciferase assay readouts are close, or otherwise a representative experiment of the two is presented.
Figure 3. Afucosylated hmstr-IgG2 and rabbit IgG exhibit enhanced ADCC via FcγRIIIA orthologues. Recombinant HM79 antibodies with Fc domains from hmstr-IgG2, hamster IgG1, or rabbit IgG, were produced by WT or Fut8−/− CHO-K1 cells. Purified WT and AF HM79 antibodies with different isotypes and fucose content all have the exact same mCD79b-recognizing Fab, but demonstrated drastic differences in ADCC assays with various Fcγ receptors. Compared with WT HM79-hmstr-IgG2, AF HM79-hmstr-IgG2 exhibits greatly enhanced ADCC via human FcγRIIIA (A), but HM79-hmstr-IgG1 antibodies had no activities (B). AF MH79-rbt-IgG is more stimulatory than WT MH79-rbt-IgG towards human FcγRIIIA (C), monkey FcγRIIIA (D) and ferret FcγRIIIA (E) in ADCC assays. Data are summarized from two independent experiments with Mean ± SD calculated from repeat experiments.

Interestingly, perhaps due to species difference, AF HM79-hmstr-IgG2 does not show any binding to rat FcγRIV (not shown). Also, compared with AF HM79-hmstr-IgG2, chimeric AF rabbit HM79-16 antibody (HM79-rbt-IgG) exhibits much weaker ADCC activities under exactly the same conditions. But still, afucosylation of rabbit IgG turns an otherwise ineffective WT antibody into a highly effective one for human, monkey and ferret FcγRIIIA (Fig. 3C–E). The chimeric pig HM79-16 antibody (HM79-pig-IgG) does not show any ADCC activities by human, monkey or ferret FcγRIIIA and mouse FcγRIV, except for pig FcγRIIIA itself where the AF pig IgG outperforms the WT counterpart (not shown). This is in line with the report that pig IgG only binds to its own FcγRIIIA but not to human or rabbit FcγRIIIA [38].

Afucosylated human IgG1 exhibits enhanced ADCC with FcγRIIIA/FcγRIV from multiple mammalian species

Having established the testing systems to functionally assign the right nomenclature to orthologues of FcγRIIIA or FcγRIV in multiple species, we now address the question whether an afucosylated therapeutic human IgG1 antibody can be directly evaluated for higher efficacy in various animal models. For that, we first used WT and AF anti-5T4 hlgG1, produced by WT and Fut8−/− CHO cells. The oncofetal antigen 5T4 (trophoblast glycoprotein, TPBG) is expressed on a wide range of human solid tumors with expression level correlated with disease progression, and is an attractive target for immune intervention in cancer [39, 40]. We have generated a humanized 5T4 IgG1 antibody with high affinity towards 5T4 antigen (Kd = 4.1 nM). In ADCC assays using h5T4-expressing CHO cells as target cells and luciferase-equipped Jurkat cells expressing various species-specific FcγRIIIA or FcγRIV as effector cells, AF anti-5T4 hlgG1 universally exhibits stronger ADCC than WT anti-5T4 hlgG1 (Fig. 4). Perhaps because our anti-5T4 antibody is already highly effective, i.e., even the WT version has EC50 numbers between 1 and 10 ng/mL for FcγRIIIA or FcγRIV from most tested species except for golden hamster, guinea pig, rabbit, cat and pig (EC50 numbers between 10 and 100 ng/mL), afucosylation of anti-5T4 enhances ADCC by only ~10-fold.

As different antibodies have different binding characteristics, to address the same question with a different antibody, we then turned to an anti-MUC1-C hIgG1 similarly produced by WT or Fut8−/− CHO cells. In ADCC assays using MUC1-expressing human colon cancer cell line HCT116 as target cells and luciferase-equipped Jurkat cells expressing various species-specific FcγRIIIA or FcγRIV as effector cells, AF anti-MUC1-C hlgG1 is highly effective whereas WT anti-MUC1-C hlgG1 does not show any ADCC activity even at the highest doses (Fig. 5), although both have the same binding profiles towards hMUC-1+ HCT116 by FACS (Supplementary Fig. S5). The enhanced ADCC in vitro by afucosylated anti-MUC1-C hlgG1 also translates into effective growth control of hMUC-1+ HCT116 solid tumor in vivo in C57BL/6 mice, whereas the WT antibody is
Figure 4. Afucosylation of an already highly potent human IgG1 (anti-h5T4) can still boost its ADCC through FcγRIIIA/FcγRIV from multiple mammalian species. Recombinant human anti-h5T4 IgG1 antibodies were produced by WT or Fut8−/− CHO-K1 cells. Compared with WT anti-5T4 hIgG1, AF anti-5T4 hIgG1 exhibits greatly enhanced ADCC via mouse FcγRIV (A), mouse FcγRIII (B), rat FcγRIV (C), Chinese hamster FcγRIV (D), golden hamster FcγRIV (E), guinea pig FcγRIV (F), ferret FcγRIIIA (G), rabbit FcγRIIIA (G1T8R84) (H), rabbit FcγRIIIA (G1T7E7) (I), cat FcγRIIIA (J), dog FcγRIIIA (K), pig FcγRIIIA (L), monkey FcγRIIIA (M) and human FcγRIIIA (N). Data are representative of two repeat experiments.

ineffective (unpublished observations). Our data strongly suggest that cross-species IgG:FcγRIIIA/FcγRIV binding profile dictates the effectiveness of the therapeutic antibody when the MOA of the antibody is through ADCC and/or ADCP. Producing afucosylated IgG by Fut8−/− CHO cells can render greater ADCC activities, and potentially allows the therapeutic effects of the antibody to be directly assessed across a wide range of animal species.
Figure 5. Afucosylation of an intrinsically weak human IgG1 (anti-MUC1-C) endows it with strong ADCC through FcγRIIIA/FcγRIV from multiple mammalian species. Recombinant human anti-hMUC1-C IgG1 antibodies were produced by WT or Fut8−/− CHO-K1 cells. WT anti-hMUC1-C IgG1 does not show ADCC even at the highest doses, whereas AF anti-hMUC1-C IgG1 exhibits greatly enhanced ADCC via mouse FcγRIV (A), mouse FcγRIII (B), rat FcγRIV (C), Chinese hamster FcγRIV (D), guinea pig FcγRIV (E), ferret FcγRIIIA (F), rabbit FcγRIIIA (G), dog FcγRIIIA (H), pig FcγRIIIA (I), monkey FcγRIIIA (J) and human FcγRIIIA (K). Golden hamster FcγRIV and cat FcγRIIIA do not respond to this particular hIgG1 even in AF form (not shown). Data are representative of two repeat experiments.

DISCUSSION

One of the biggest hurdles in biomedical research is the translatability of animal studies in the context of human disease settings. At the cellular level, naïve lab animals (usually mice) used at young ages have T and B cell repertoires very different from those in a human being where antigen-experienced memory lymphocytes also constitute and regulate immune responses in many unique ways [41]. At the molecular level, the species
differences in the interacting Ag/Ab and ligand/receptor pairs make result interpretation not entirely without reservation. Transgenic or humanized knockin animals provide only part of the solution, as the challenges posed by the convoluted networks of ligand:receptor interactions, esp. within the immune system, are beyond the current technologies of making genetically-modified animals can offer to overcome. Generating hematopoietic chimeric animals with human immune cells is a viable strategy, but these animals are extremely expensive, and the technology is not widely applicable other than in our little furry friends called Mus musculus. Yet, many underrepresented mammalian species, such as hamster, guinea pig, ferret, cotton rat, pig (including minipigs), rabbit and marmoset are increasingly being used as human disease models, especially in the infectious disease area. The technologies suitable for evaluating antibody therapeutics and studying their MOAs have not been adequately applied in these underrepresented species.

This study was prompted during our attempt of investigating a therapeutic human IgG1 fusion protein in the hamster model of SARS-CoV-2 infection, where we were puzzled whether afucosylated hIgG1 could still exhibit enhanced ADCC and achieve better therapeutic effects in hamster. In fact, throughout the literature, there lacks not only a systematic functional study to address this crucial question across many species, but also the careful consideration of orthologues of human FcγRIIIA in these species in the first place. We performed exhaustive BLAST searches and used bioinformatics tools to narrow down the potential orthologues of human FcγRIIIA or mouse FcγRIV from rat, hamster, guinea pig, ferret, rabbit, cat, dog, pig and monkey, some of which are only newly characterized in this paper. For instance, prior to this study, the existence of ferret FcγRIIIA was merely predicted by automated computational analysis, but the receptor itself has not been functionally studied. We found that ferret FcγRIIIA is most closely related to dog FcγRIIIA in the phylogenetic tree (Fig. 1B), and confirmed that these two receptors exhibit very similar response patterns towards the 5F2 mIgG2c/mIgG1 combo set (Fig. 2G and K). Likewise, even though the two FcγRIV receptors from Chinese hamster and golden hamster are as disparate as FcγRIV receptors from mouse and rat in the phylogenetic tree (Fig. 1B), they are still orthologues to each other, not different genes. Chinese hamster and golden hamster do have a different set of FcγRIII receptors, similar to those found in mouse and rat (Supplementary Fig. S2). Interestingly, FcγRII and FcγRIV receptors from guinea pig are only remotely related to the other rodent family members (Supplementary Fig. S2). Although the FcγRIV receptors from mouse, rat and hamster all have in their intracellular domains the YEEP motif, whose tyrosine residue becomes phosphorylated after receptor ligation, gpFcγRIV has the KEED sequence and all the FcγRIIIA receptors from non-rodent species do not have this YEEP signature (Fig. 1A). Thus, tyrosine phosphorylation at this position is not absolutely required, as signaling of FcγRIIIA and FcγRIV is mediated by the Fc receptor common gamma chain [42], which is co-expressed in all our Jurkat reporter cell lines.

With the sensitive luciferase-based ADCC assay, we are now confident to conclude that IgG afucosylation can be a universal method to enhance ADCC/ADCP mediated by the orthologues of human FcγRIIIA or mouse FcγRIV across multiple mammalian species, most likely not limited to those in our current study. For research and development of antibody therapeutics, IgG afucosylation can be achieved via two major routes. First, if one has generated a hybridoma-derived MAb, which has an isotype of ADCC potential, such as mouse IgG2a/b from BALB/c or mIgG2b/c from C57BL/6, then one can use CRISPR technology to directly knock out the Fut8 gene in the existing hybridoma cell line to produce the afucosylated antibody (Fig. 2). If the hybridoma is of IgG1 isotype, although one can perform in vitro class switching to IgG2a/b/c, the conditions are tricky and the efficiency could be very low [43]. Second, and most appealing from the pharmaceutical industry perspective, one can clone the MAb's VH/VL genes from the hybridoma mRNA by RT-PCR, and recombinantly express the original or humanized antibody with the desired isotype and afucosyl form in Fut8− CHO cells (Figs 3–5).

To our surprise, in the ADCC assays, simple afucosylation can transform an otherwise ineffective IgG antibody into a highly active one with potent ADCC activities in certain species. For instance, WT mIgG2c barely triggers any ADCC by hamster FcγRIV, or by rabbit and cat FcγRIIIA, whereas AF mIgG2c is highly effective (Fig. 2). The same is also true for a hamster-IgG2 and a rabbit IgG (Fig. 3), as well as a hIgG1 (Fig. 5). Therefore, as an economic way to tap into the vast pool of hybridoma-derived antibodies from academia labs or biotech companies for pre-clinical studies of candidates with therapeutic potential, one should always bear in mind to: 1) select or switch to an ADCC-capable isotype, and 2) generate its afucosylated form.

A good example is when we were approached by our academic collaborators on how to evaluate a mouse CD39 antibody (clone 5F2) for any anti-tumor effect. Its mIgG1 form had been extensively examined by the collaborators in B16F10 mouse melanoma model, and was found to be completely ineffective. We first tried to recombinantly express AF 5F2-mIgG2c in Fut8− CHO cells, but 5F2 antibody genes belong to that “difficult-to-express” category, and this approach was not successful. Fortuitously, using ISO-M8c mouse immunoglobulin isotyping kit specifically formatted for MAbs of C57BL/6 origin, we spotted in the culture supernatant the presence of 5F2’s spontaneously class-switched mIgG2c form and subeloned this hybridoma subline. This was followed by knocking out the murine Fut8 gene in the WT 5F2 mIgG1 and mIgG2c hybridomas, with the ultimate ensemble of WT and AF 5F2 mIgG1/mIgG2c combo to test our hypothesis. In both B16F10 melanoma and MC38 colon cancer models, and reminiscent of their dramatic differences in ADCC capacities (Fig. 2A), AF 5F2-mIgG2c demonstrated most striking anti-tumor effect, followed by WT 5F2-mIgG2c, whereas 5F2-mIgG1 antibodies were completely ineffective (manuscript in preparation, US provisional patent application 62/685 176).

With such dramatic in vivo outcomes directly correlated with in vitro ADCC activities, we are confident that the
observations in our study can provide valuable insights for researchers to break the boundary and explore possibilities not ever imagined before. For example, as global concerns arise as the emerged and rapidly spreading SARS-CoV-2 variants might escape host immunity induced by vaccination, researchers have isolated rabbit MAbS with potent neutralization capability against not only SARS-CoV-2 WT strain but also emergent variants [44]. The longer CDR3 of rabbit IgG permits these antibodies to bind inside clefts and grooves that are generally inaccessible to conventional antibodies and make them great candidates for binding within enzyme active sites and other “hard to reach” protein surfaces. Based on our results, afucosylated rabbit IgG exhibits much potent ADCC via ferret FcγRIIIA than WT rabbit IgG, which is ineffective (Fig. 3E), it is now possible to test the hypothesis in the ferret model of SARS-CoV-2 infection whether afucosylated rabbit IgG can be more protective not only through neutralization but also through ADCC-mediated clearing of virus-infected cells. Conversely, by comparing adoptively transferred WT and AF anti-Spike/RBD IgG side-by-side in the ferret model of SARS-CoV-2 infection, scientists may acquire valuable insights whether disease is aggravated via enhanced stimulation through FcγRIIIA/FcγRIV by afucosylated IgG that may trigger host cytokine storm [16, 17]. If that is the case, counter measures can be developed to treat severe illness in COVID-19.

In summary, our findings indicate that increased sensitivities of FcγRIIIA/FcγRIV to afucosylated IgG with enhanced ADCC is a universal cross-species phenomenon, underlining the rationale of testing afucosylated therapeutic antibodies directly in various animal models for efficacy and toxicity evaluation. Not only this strategy could speed up antibody discovery and help save tremendous resources in animal studies, perhaps it could also turn someone’s trash into another one’s treasure, and nurture the next generation glycoengineered therapeutic antibodies with novel mechanisms of disease-fighting actions.

AUTHORS’ CONTRIBUTIONS
C.M. performed most experiments, including the ADCC assay, FACS and the animal studies and analyzed the data. R.N. made lentiviruses and established Jurkat cell lines for the ADCC assay. X.Z. and W.G. designed the experiments, wrote the animal protocols, monitored and financially supported the study. W.G. wrote the manuscript. All authors critically reviewed the drafts of the manuscript and approved the final version.

SUPPLEMENTARY DATA
Supplementary data are available at ABT online.

ACKNOWLEDGEMENTS
The authors would like to thank Dr Simon C. Robson from Beth Israel Deaconess Medical Center for the original anti-mCD39 hybridoma 5F2, and Genus Oncology, LLC. for anti-MUC1-C antibody.

DATA AVAILABILITY STATEMENT
The data underlying this article are available in the article and in its online supplementary material.

CONFLICTS OF INTEREST STATEMENT
C.M. and W.G. are employees of Antagen Pharmaceuticals, Inc., which owns a proprietary Fut8−/− CHO cell expression system.

FUNDING
This study was supported by National Institutes of Health (NIH) Small Business Innovative Research grants (HHSN272201800017C, 75N93019C00014 and 75N93020C00042) awarded to Antagen.

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