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1 **An Engineered Human Fc Domain that Behaves like a pH-Toggle**
2 **Switch for Ultra-Long Circulation Persistence**

3
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33

34 **Abstract**

35 The pharmacokinetic properties of antibodies are largely dictated by the pH-
36 dependent binding of the IgG fragment crystallizable (Fc) domain to the human neonatal
37 Fc receptor (hFcRn). Engineered Fc domains that confer a longer circulation half-life by
38 virtue of more favorable pH-dependent binding to hFcRn are of great therapeutic interest.
39 Here we developed a pH Toggle switch Fc variant containing the L309D/Q311H/N434S
40 (DHS) substitutions, which exhibits markedly improved pharmacokinetics relative to both
41 native IgG1 and widely used half-life extension variants, both in conventional hFcRn
42 transgenic mice and in new knock-in mouse strains. engineered specifically to recapitulate
43 all the key processes relevant to human antibody persistence in circulation, namely: (i)
44 physiological expression of hFcRn, (ii) the impact of hFcγRs on antibody clearance and (iii)
45 the role of competing endogenous IgG. DHS-IgG retains intact effector functions, which are
46 important for the clearance of target pathogenic cells and also has favorable developability.

47

48 **Introduction**

49 IgG isotype antibodies avoid clearance by endolysosomal degradation by virtue of
50 their pH-dependent binding to the neonatal Fc receptor (FcRn), which is expressed by cells in
51 nearly every organ in mammals¹⁻³. The FcRn receptor consists of the glycosylated heavy α -
52 chain polypeptide, an MHC I class family member which associates with β 2-microglobulin
53 (β 2m)^{4,5}. IgG internalized by pinocytosis binds to FcRn at endosomal pH (5.5-6.0) and as a
54 result, instead of being directed to the endolysosomal compartment for degradation,
55 IgG:FcRn complexes are sorted into tubules originating from sorting endosomes and directed
56 to return to the plasma membrane. Upon fusion with the plasma membrane, the intracellular
57 fluid within the tubules is released and rapidly equilibrates with the extracellular pH 7.4⁶⁻⁸.
58 At extracellular pH, the affinity of FcRn for the Fc domain is so low that antibodies are
59 released back into circulation. This process occurs readily, despite the strong avidity effects
60 of both the high local concentration of FcRn at the site of vesicle fusion, and the 2:1
61 stoichiometric binding of FcRn to IgG^{9,10}. Biophysical studies have elucidated the molecular
62 details of the IgG:FcRn interaction including the role of residues in the CH2-CH3 interface of
63 the Fc domain in contact with FcRn, the pivotal role of His310 and His435 on pH-dependent
64 binding, and the significance of protein dynamics¹¹⁻¹³.

65 Engineering the human Fc domain to improve pharmacokinetic (PK) properties,
66 (manifest as an increased area under the plasma drug concentration-time curve (AUC), a
67 lower clearance rate and a longer β phase $T_{1/2}$ ^{14,15}) is of great interest for therapeutic
68 purposes^{3,16-20}. Better pharmacokinetics enable less frequent administration and lower dosing,
69 which in turn translate into improved patient compliance and lower costs. Mutations of the Fc
70 domain that enhance the affinity for FcRn at both the endosomal and physiological pH have
71 been shown to result in greater antibody clearance. In contrast, Fc mutations that

72 preferentially enhance FcRn affinity at pH 5.8 confer increased antibody half-life in
73 circulation^{16,18,20-23}. Fc domains containing the amino acid substitutions M428L/N434S (LS
74 mutant), M252Y/S254T/T256E (YTE mutant), or H433K/N434F (KF mutant) confer 10- to
75 12-fold higher affinity for FcRn at pH 5.8, result in the greatest reported increase in antibody
76 half-life (2- to 4- fold in circulation) in mice and in non-human primates, and are currently
77 being evaluated in multiple clinical trials^{16,24-26}. Notably, ravulizumab, a complement C5-
78 inhibiting antibody containing the LS mutations, was FDA-approved for paroxysmal
79 nocturnal hemoglobinuria and shown to have a serum half-life of ~49.7 days²⁷, and
80 MEDI8897, a respiratory syncytial virus (RSV)-neutralizing antibody containing the YTE
81 mutations, was shown to have a serum half-life of ~70 days, compared to approximately 20
82 days for wild type (wt) IgG1, in infants in a phase 1b/2a study for prophylaxis against RSV
83 infection²⁸.

84 While further improvements in human antibody pharmacokinetics are highly
85 desirable, extensive protein engineering campaigns focused on identifying mutations that
86 confer even higher affinity for FcRn at pH 5.8 relative to the YTE or LS Fc variants have so
87 far failed to yield amino acid combinations capable of imparting even longer circulation half-
88 life in animal models^{20,23,29-31}. Notably, YTE- and LS- Fc domains have significant binding to
89 FcRn at pH 7.4 under high avidity conditions¹¹. Residual binding at pH 7.4 may have a
90 negative effect on plasma recycling and circulation persistence^{32,33}. Another key
91 consideration in the engineering of antibodies with ultra-long circulation half-life is that any
92 mutation introduced into the Fc domain, should not negatively impact other antibody
93 characteristics such as effector functions or manufacturability³⁴. For example, the YTE, LS
94 and KF mutations reduce FcγR binding and effector functions to various degrees (depending

95 on the mutant antibody and patient Fc γ R allotype), and the YTE mutations have been
96 reported to also reduce complement dependent cytotoxicity (CDC)^{18,35}.

97 Antibody pharmacokinetics are routinely evaluated in transgenic (Tg) mice
98 overexpressing human FcRn (hFcRn) from non-native promoters^{36,37}. However, the currently
99 available hFcRn Tg mouse models do not recapitulate many of the mechanisms that affect
100 antibody clearance. Key limitations of the existing mouse models used for antibody
101 pharmacokinetic studies arise from: (i) non-physiological levels of transgene hFcRn
102 expression relative to mouse FcRn in wild-type animals³⁸; (ii) expression of mouse β 2m
103 instead of human β 2m (h β 2m) in many of the currently used models^{36,37,39}; (iii) absence of
104 endogenously produced human IgG to compete for hFcRn binding⁴⁰; and (iv) no ability to
105 account for the effect of binding to human Fc γ Rs in clearance^{41,42}. To address these
106 limitations, as part of this work we constructed new knock-in mouse models that express
107 hFcRn, h β 2m, and hFc γ Rs; and, in addition, produce endogenously chimeric mouse-human
108 IgG1, composed of mouse variable regions and human constant regions for the heavy and
109 light chain (hIgG1, κ).

110 Here, we report the engineering of a human IgG Fc domain which, by virtue of having
111 moderately higher affinity for FcRn at pH 5.8 but no detectable binding at pH 7.4 under high
112 avidity conditions, confers improved antibody PK properties compared to the clinical stage
113 YTE and LS variants, both in the commonly used hFcRn^{Tg} mouse model (hemizygotic 276)
114 as well as in our new knock-in model (hFcRn-h β 2m-hFc γ Rs-hIgG1, κ mice). Importantly, we
115 show that antibodies utilizing our engineered ultra-long half-life Fc domain display the full
116 range of effector functions as the wt Fc domain while exhibiting far favorable biophysical
117 properties for clinical development.

118

119 **Results**

120 **Pharmacokinetics properties of an engineered human Fc variant:** We developed a
121 strategy that capitalizes on *E.coli* display of large combinatorial IgG libraries (Anchored
122 Periplasmic Expression (APEX) technology⁴³⁻⁴⁶) for the isolation of clones expressing human
123 IgG1 with mutated Fc domains that bind selectively to the human FcRn/human β 2m complex
124 (hFcRn:h β 2m) at pH 5.8 but not at pH 7.4 (**Fig. 1a**). Select regions of the human IgG1 C_H2-
125 C_H3 hinge were combinatorially mutagenized (**Supplementary Fig. 1a-c** and
126 **Supplementary Table 1**) to create a library of $>10^8$ transformants. Briefly, *E. coli*
127 spheroplasts expressing mutated human IgG1 (Trastuzumab) anchored on the external leaflet
128 of the inner membrane were first screened by FACS for binding to Alexa488-labelled
129 hFcRn:h β 2m. Three rounds of FACS with hFcRn:h β 2m at pH 5.8 were performed to enrich
130 antibodies with Fc domains with higher binding affinity at pH 5.8. The pool of clones
131 enriched for enhanced binding at endosomal pH was then subjected to a competitive, two-
132 step labeling process to eliminate variants that have detectable binding at pH 7.4 to high
133 avidity, dimeric, GST-hFcRn:h β 2m. For this purpose, spheroplasted cells were labelled with
134 an excess of Alexa647-labeled GST-hFcRn:h β 2m (red) at pH 7.4 and then spheroplasts were
135 washed with pH 7.4 PBS. The spheroplasts were subsequently labelled with monomeric
136 hFcRn:h β 2m-Alexa488 (green) at pH 5.8, and clones with high green fluorescence and low
137 red fluorescence (i.e. absence of residual Alexa647-labeled GST-hFcRn:h β 2m from the first
138 labeling step) were isolated and characterized (**Fig. 1a**). Four clones expressing different Fc
139 variants from the last round of screening were isolated, individually confirmed to display pH-
140 dependent binding by FACS and were all found to share three amino acid substitutions:
141 V264E, L309D, and Q311H (EDH) (**Supplementary Fig. 1d**).

142 The four isolated antibody variants were produced in HEK293 cells. One variant,
143 EDHY-IgG1 (V264E, L309D, Q311H, and N434Y) had a ~20-fold better affinity ($K_{D, pH5.8} =$
144 28 nM) for hFcRn at pH 5.8 compared to wt IgG1 ($K_{D, pH5.8} = 550$ nM) but also had residual
145 binding at pH 7.4. A second variant, EDHS-IgG1 (V264E, L309D, Q311H, and N434S) had
146 a ~6-fold increased affinity at 5.8 ($K_{D, pH5.8} = 93$ nM) but no apparent binding at pH 7.4
147 (**Supplementary Fig. 1e-g**). Very extensive mutagenesis efforts aimed at identifying
148 mutations conferring even better $K_{D, pH 5.8}$ were unsuccessful, as all of the mutants isolated
149 based on higher affinity at pH 5.8 had detectable binding at pH 7.4 based on FACS analysis.

150 Antibodies with the EDHS mutations in the Fc had drastically reduced binding to
151 effector hFcγRs (**Supplementary Fig. 1h**); which was found to be due to the V264E
152 mutation⁴⁷. Since a V264E substitution has a minimal effect on hFcRn binding at pH 5.8⁴⁸, it
153 was reverted back to a valine to yield the L309D/Q311H/N434S (DHS) Fc domain, resulting
154 in complete recovery of hFcγR binding (**Supplementary Fig. 1h**). Moreover, these three
155 amino acids substitutions did not affect the N-glycosylation pattern of the Fc domain which
156 was comparable to that of wt IgG1 antibodies (**Supplementary Fig. 2**).

157 We performed a detailed analysis of the hFcRn:hβ2m binding kinetics of DHS
158 antibodies as a function of pH using SPR analysis with immobilized hFcRn:hβ2m at low,
159 medium or high density¹¹ (500, 2,000 or 4,000 RUs, **Fig. 1b-d** and **Supplementary Fig. 3**).
160 Consistent with earlier reports⁴⁹, YTE- and LS-IgG1 showed moderate and significant
161 binding, at pH 7.4, respectively when SPR analysis was performed with medium or high
162 hFcRn:hβ2m densities (**Fig. 1b-c** and **Table 1**). In contrast, we could detect no binding of
163 DHS- or wt-IgG1 to hFcRn:hβ2m at physiological conditions at even the highest density
164 tested (**Fig. 1b**).

165 The pharmacokinetics of Trastuzumab, which does not bind any mouse targets,
166 formatted with DHS-, YTE-, LS- or wt-IgG1 Fc domains were first evaluated in hemizygous
167 hFcRn^{Tg} mice (hemizygotic Tg276), a widely used Tg mouse model for comparing the
168 circulation persistence of human antibodies³⁷. Following administration of a single
169 intravenous dose (i.v. 2 mg/kg) by tail vein injection, the β phase $T_{1/2}$ (β phase half-life) of
170 DHS was 2.0- and 3.1-fold higher compared to YTE and LS variants (290.9 ± 25.6 h
171 compared to 148.4 ± 36.8 h and 92.9 ± 6.1 h, respectively, data are presented as mean \pm
172 standard deviation). Total drug exposure over time for DHS-IgG1 (AUC_{inf}) was 1.6-, 1.9- and
173 5.3-fold greater relative to YTE-, LS- and wt-IgG1 variants, respectively (**Fig. 1d** and **Table**
174 **2**). Antibody clearance followed biphasic kinetics, with a clearance rate of 0.11 ± 0.01
175 ml/day/kg for DHS-IgG1, approximately 6-fold lower than wt IgG1, and 1.9- and 2.2-fold
176 lower than YTE- and LS-IgG1. Introduction of the DHS mutations into the Fc domain of
177 IgG2, IgG3 (G3m16 allotype with H435 residue) or IgG4 subclasses also resulted in no
178 detectable interaction with FcRn at pH 7.4, and likewise, conferred significantly improved
179 pharmacokinetics for all the other three IgG subclasses (**Fig. 1e-g**, **Supplementary Fig. 4**,
180 and **Supplementary Table 2**). Collectively, our data: (a) reveal that the DHS Fc domain
181 imparts improved antibody PK properties in the hemizygous Tg276 hFcRn^{Tg} model relative
182 to the best in class clinical stage, half-life extension variants YTE and LS and (b) support the
183 hypothesis^{32,33} that residual binding to FcRn at physiological pH has a major detrimental
184 effect on antibody circulation persistence.

185 **Pharmacokinetic studies in novel knock-in mouse models:** To more accurately
186 evaluate antibody pharmacokinetics we constructed knock-in (KI) mouse models that account
187 for multiple processes known to affect human IgG clearance and homeostasis (**Fig. 2a-e**). In
188 these KI mouse strains: (i) the hFcRn:h β 2m complex is expressed in a manner that faithfully

189 reflects mouse FcRn levels, (ii) the human effector FcγR family is expressed to account for
190 FcγR-dependent clearance and finally, (iii) human IgG1, which, unlike murine IgG, competes
191 with administered therapeutic antibodies for hFcRn binding⁴⁰, is produced endogenously. We
192 developed a mouse in which the murine FcγR genes had been exchanged with the
193 corresponding human genes, including introns and regulatory elements for *FCGR1A* and for
194 *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B* genes (hFcγR^{KI} mouse, intercross of
195 VG6074 and VG1543 mice⁵⁰, **Fig. 2a-b**)⁵¹. The *Fcgrt* (mouse FcRn gene) was replaced with
196 *FCGRT* (human FcRn gene, VG1481, **Fig. 2c**) and the *B2m* (mouse β2m gene) was replaced
197 with *B2M* (human β2m gene, VG5153, **Fig. 2d**). The hFcRn^{KI} hβ2m^{KI} hFcγR^{KI} mice
198 (designated as Marlene mice) were bred by crossing the four transgenes as shown in **Fig. 2e**.
199 Finally, to account for the fact that endogenous mouse IgG does not compete with human IgG
200 for binding to human FcRn⁵², the heavy-chain constant region of human IgG1 (*IGHG1*) was
201 knocked-in to replace the switch μ region of the mouse heavy-chain locus, and the human
202 kappa light-chain constant region (*IGK*) was knocked-in to replace the mouse kappa light-
203 chain region (kappa light chains are used in 95% of circulating antibodies in mice), thereby
204 generating mice that exclusively produce human IgG1 heavy chains, predominantly
205 associated to human kappa light chains (**Fig. 2f-g**). The hFcRn^{KI} hβ2m^{KI} hFcγR^{KI} hIgG1,κ^{KI}
206 mice (designated as Scarlett mice) were bred by crossing the Marlene and the hIgG1,κ^{KI} mice
207 (**Fig. 2h**). hFcRn expression was confirmed by immune-histochemistry and RT-PCR of
208 spleens of Scarlett mice (**Fig. 2i** and **Supplementary Fig. 5**). Cell type-specific expression of
209 hFcγRs in Marlene and Scarlett is reported in **Fig. 2j** and **Supplementary Fig. 6** is similar to
210 that of humans, and consistent with our earlier reported expression pattern for hFcγR^{KI}
211 mice⁵⁰. Scarlett mice displayed 440 ± 120 μg/mL of human IgG1 in serum and undetectable

212 levels of mouse IgG. The mouse IgG serum concentration in control C57BL/6J mice was 605
213 ± 147 $\mu\text{g/mL}$, in the same order of magnitude as human IgG1 in Scarlett mice.

214 We observed very comparable β -phase elimination half-life in the KI animals that
215 produce endogenous human antibodies (Scarlett) for three different approved IgG1 antibodies
216 (Trastuzumab, Rituximab, or Omalizumab) after a single intravenous dose (i.v. 2 mg/kg) by
217 tail vein injection. Specifically, for Trastuzumab we compared the pharmacokinetics
218 between the Scarlett mice and animals that do not have circulating human IgG1 (Marlene
219 mice; **Supplementary Fig. 7**). The β -phase elimination half-life was 40% lower in the
220 Scarlett mice underscoring how competition for FcRn binding by circulating human IgG1
221 impacts the pharmacokinetics of administered antibodies in hFcRn^{Tg} mice.

222 Consistent with the trends observed in the hemizygous Tg276 hFcRn^{Tg} mouse model,
223 the data in **Fig. 2k** and **Table 3** reveal that in Scarlett mice - DHS antibodies have $\geq 50\%$
224 longer β phase half-life ($P \leq 0.0001$ to LS- or YTE-IgG1), a proportional increase in AUC_{inf}
225 ($P \leq 0.0001$ to LS- or YTE-IgG1), and a proportional decrease in clearance rate ($P \leq 0.0001$
226 to LS- or YTE-IgG1) relative to the YTE and LS variants. Compared to wt IgG1, the DHS
227 mutations resulted in 4-fold longer β phase half-life and 5-fold greater AUC_{inf} .

228 **Effector Functions and Biophysical Properties of DHS-Fc:** Mutations that
229 favorably impact antibody serum half-life can adversely affect binding to effector hFc γ Rs, in
230 turn impairing the clearance of target cells by ADCC or ADCP³⁵. Consistent with earlier
231 reports, SPR measurements showed that the YTE mutations reduce binding to the low affinity
232 Fc γ Rs (i.e. hFc γ RIIa, hFc γ RIIb, hFc γ RIIIa) by more than 3-fold. The LS substitutions have a
233 more modest effect on hFc γ R binding, resulting in a 2-fold reduction in affinity for hFc γ RI
234 and hFc γ RIIa_{H131}, and 3-fold reduction for hFc γ RIIIa_{V158}. In contrast, the affinities of the

235 DHS-IgG1 for hFcγRs were statistically indistinguishable (or slightly improved in the case of
236 hFcγRIIIa_{F158}) compared to those of wt-IgG1 (**Table 4** and **Supplementary Fig. 8a**). Class-
237 switched IgG2, IgG3 or IgG4 variants of Trastuzumab bearing DHS mutations also showed
238 statistically indistinguishable binding to hFcγRs relative to their wt counterparts
239 (**Supplementary Fig. 8b**). Not surprisingly, the *in vitro* ADCC activity of DHS-Trastuzumab
240 IgG1 was identical to that observed with wt Trastuzumab in hFcγRIIIa-*V/V* and -*V/F* donors,
241 but greater than that of YTE-Trastuzumab IgG1 or LS-Trastuzumab IgG1 (**Fig. 3a**). On the
242 other hand, as expected³⁵, with homozygotic hFcγRIIIa-*F/F* donors, DHS-Trastuzumab IgG1
243 showed equivalent ADCC activities with wt Trastuzumab or LS-Trastuzumab IgG1 (**Fig. 3a**).

244 Next, we examined the ability of anti-CD20 (Rituximab) antibody variants to activate
245 the classical complement pathway via the recruitment of C1q. Target cells (CD20⁺ Raji)
246 opsonized with DHS-Rituximab IgG1 antibodies had a higher level of cell surface-deposited
247 C1q relative to wt-, LS- or YTE-Rituximab antibodies. DHS-Rituximab displayed 2-fold
248 more potent CDC ($0.7 \pm 0.1 \mu\text{g/ml}$) than wt- ($1.5 \pm 0.1 \mu\text{g/ml}$) or LS-Rituximab (1.9 ± 0.1
249 $\mu\text{g/ml}$), and 10-fold more potent CDC than YTE-Rituximab, the latter which has already been
250 reported to display significantly lower CDC activity than wt-Rituximab^{18,48} (**Fig. 3b-c**).

251 Rheumatoid factor (RF) refers to auto-antibodies that bind to the Fc domain of
252 immunoglobulins. The binding of RF to therapeutic antibodies is associated with faster
253 clearance and increased antigen presentation and anti-drug antibodies⁵³⁻⁵⁶. We measured the
254 RF binding of the wt-, DHS-, YTE- and LS-IgG1 by ELISA using polyclonal sera from
255 donors with high RF titers. DHS-IgG1 showed binding to RF sera that was indistinguishable
256 from that of wt-IgG1 and significantly lower than that observed with YTE-IgG1 and
257 especially with LS-IgG1 (**Fig. 3d**).

258 Size exclusion chromatography (SEC) showed that for all 4 IgG subclasses, introduction of
259 the DHS mutations did not affect protein solubility, nor did it lead to soluble aggregate
260 formation (**Supplementary Fig. 9a**). The thermodynamic stability of therapeutic antibodies
261 is one of the most important biophysical properties for clinical development^{34,57,58}. The
262 melting temperature (T_m) was comparable for the wt, DHS, and LS³⁵ Fc variants (**Table 5**)
263 but was more than 6°C lower for the YTE variant (**Table 5**)³⁴. The susceptibility of the
264 antibody variants to aggregation at 70°C was very high for YTE-IgG1, leading to visible
265 precipitates within minutes, intermediate for LS-IgG1 and minimal for wt- and DHS-IgG1
266 (**Supplementary Fig. 9b-e**). Similarly, the YTE- and LS- IgG1 formed higher levels of
267 soluble aggregates upon prolonged incubation at 50 °C (**Fig. 3e**). Finally, the self-association
268 propensity of the various engineered Fc antibodies at room temperature was determined by
269 affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS) a widely used assay for
270 antibody developability⁵⁸⁻⁶⁰. Earlier AC-SINS analyses using 137 FDA-approved antibody
271 drugs, found that AC-SINS plasmon shifts >11.8 nm correspond to antibodies with high
272 levels of self-association and which represents a development risk⁵⁸. For DHS-IgG1 the AC-
273 SINS plasmon shift was only 0.1 ± 0.5 nm; of note, YTE- and LS- antibodies also showed
274 low plasmon shift in this assay albeit higher than that of DHS-IgG1 (**Fig. 3f**).

275

276 DISCUSSION

277 An ideal Fc domain for therapeutic applications must confer optimal pH dependent
278 binding to hFcRn to achieve very long circulation half-life, maintain intact effector functions
279 and display favorable biophysical properties suitable for clinical development^{57,58}. To enable
280 maximal antibody circulation half-life, an Fc domain should resemble a pH toggle switch
281 with a low FcRn $K_{D\text{ pH }5.8}$ and no detectable binding activity at pH 7.4. However, despite
282 extensive efforts, engineering Fc domains with a lower FcRn $K_{D\text{ pH }5.8}$ and no detectable
283 binding at pH 7.4 under high avidity conditions had proven elusive^{11,20}. Here, we used a two-
284 step high-throughput screening strategy to isolate the DHS Fc variant, which has a 5-fold
285 lower $K_{D\text{ pH }5.8}$ for FcRn relative to wt-IgG1 Fc and no detectable binding at pH 7.4. Unlike
286 antibodies containing the YTE or LS half-life extension mutations, the DHS Fc domain does
287 not bind to high densities of immobilized hFcRn at pH 7.4 (**Fig. 1b-c** and **Table 1**). We found
288 that, by virtue of the lack of detectable binding at pH 7.4 and despite having a lower affinity
289 for FcRn at endosomal pH relative to the LS or YTE variants, DHS antibodies exhibit even
290 more favorable pharmacokinetics in multiple mouse models. Specifically, as shown in **Fig.**
291 **1d** and **Table 2**, the DHS Fc domain confers significantly improved antibody
292 pharmacokinetic properties relative to the YTE and LS variants in hemizygous Tg276 mice, a
293 widely used model for antibody PK studies³⁷. In the Tg276 model, hFcRn is transcribed from
294 the CAG promoter, resulting in ubiquitous high-level gene expression³⁶. A second transgenic
295 mouse line, Tg32, in which hFcRn is under the control of the human hFcRn promoter, is also
296 commonly used for antibody PK studies⁴⁶; however, in this model the transgene is inserted in
297 close proximity to a mouse enhancer region which in turn impacts transcription and tissue-
298 specific expression. Because neither model fully recapitulates mouse endogenous FcRn
299 expression³⁸, we constructed new models in which hFcRn and h β 2m are expressed from the

300 respective mouse promoters. The binding of antibodies to FcγRs, and in particular to FcγR3
301 expressed by liver-resident cells are known to affect antibody clearance^{41,42}. However, since
302 human antibodies bind to mouse FcγRs with very different affinities than those of their
303 human counterparts^{61,62} the effect of FcγR-mediated clearance of human IgG cannot be
304 properly accounted for in animals expressing murine IgG receptors. To address this
305 shortcoming, we further engineered hFcRn^{KI} hβ2m^{KI} mice to also express all the human FcγR
306 family proteins (Marlene mice). Yet another factor that plays an important role for
307 therapeutic antibody pharmacokinetics is the competition for hFcRn binding by endogenous,
308 circulating human IgG. Because mouse IgG does not bind to hFcRn⁴⁰ in some earlier
309 antibody PK studies in Tg276 or Tg32 hFcRn^{Tg} mice intravenous co-administration of IgG
310 (IVIG) was attempted as a surrogate for endogenous IgG competition. However, the effect of
311 IVIG was reported to be dependent on the mouse model and was more pronounced in animals
312 expressing lower hFcRn levels^{40,63}. To overcome these problems, we further engineered the
313 Marlene mice to express chimeric mouse-human IgG1 containing the constant regions of
314 human IgG1 heavy chain and human kappa light chain. The resulting Scarlett mouse strain
315 displayed circulating human IgG1 concentrations comparable to those of mouse IgG1 in
316 C57BL/6J mice. We note however that even though the Scarlett model produce levels of
317 human IgG1 comparable to what is physiologically relevant for mouse IgG1, it may
318 nonetheless somewhat underestimate the impact of competing IgG1 in humans due to species
319 differences since humans produce higher levels of this antibody isotype. . Beyond their utility
320 in comparing the PK of different half-life extension variants as shown here, these new knock-
321 in models are likely to prove particularly useful for preclinical studies for the understanding
322 of antibody effector functions in mice that express physiologically relevant levels of hFcγRs
323 and additionally express competing endogenous IgG1.

324 Consistent with the improved PK properties of antibodies with the DHS mutations
325 compared to antibodies with the clinical-stage YTE and LS variants in Tg276 hemizygous
326 mice in **Fig. 1d** and **Table 2**, the DHS amino acid substitutions resulted in $\geq 50\%$ increase β
327 phase $T_{1/2}$ and AUC_{inf} in the Scarlett model. The relative improvement in the PK parameters
328 of DHS antibodies compared to the YTE or LS variants in the Scarlett model was lower than
329 the effect (2-fold) observed in Tg276 and may be due to competition by IgG, the impact of
330 effector Fc γ R clearance mechanisms and possibly, mouse strain background differences. If,
331 as expected, the pharmacokinetic behavior of antibodies in Scarlett mice more closely mimics
332 that in humans, the observed 50% greater β -phase half-life and drug exposure afforded by the
333 DHS mutations should result in significant therapeutic benefit. For example, a 50%
334 improvement over YTE antibodies²⁸ could be expected to translate into a circulation half-life
335 in humans of well over 100 days.

336 To quantitatively explore the significance of endosomal retention (determined by K_D
337 at pH 5.8) versus surface recapture (determined by the K_D at pH 7.4) we constructed a simple
338 phenomenological PK model (**Supplementary Fig. 10**). This model is sufficient to explain
339 the relative PK properties of each Fc domain. Sensitivity analysis indicates that surface
340 recapture is the dominant parameter limiting circulation persistence of YTE- and LS-IgG1
341 mutants. Specifically, in the hemizygotic Tg276 mice as well as in the Scarlett mice, the
342 consequence of residual affinity of these Fc domains at the extracellular pH is roughly 50%
343 or 20% recapture, respectively, of antibody on the cell surface during endosomal recycling.
344 Collectively, both experimental PK comparison and the quantitative modeling of antibodies
345 with distinct hFcRn binding properties at endosomal and physiological pH highlight the
346 negative effect of hFc:hFcRn binding at pH 7.4 on antibody circulation persistence.

347 Fc-mediated effector functions (ADCC and CDC), developability (biophysical
348 properties including thermodynamic stability), and drug bioavailability (serum half-
349 life/AUC_{inf}) are of critical importance for clinical application of therapeutic antibodies^{58,64}.
350 We demonstrate herein that the DHS Fc variant has (i) intact Fc-mediated effector functions,
351 (ii) a similar T_m, low aggregation propensity, no evidence for self-association and low
352 binding to rheumatoid factor, similar to wt IgG1 antibodies, and (iii) ultra-long circulation
353 persistence in highly relevant new humanized mouse models.
354

355 MATERIALS AND METHODS

356 Cells and Reagents

357 Adenocarcinoma SK-BR3 cell line (ATCC[®] HTB-30[™]) and Raji cell line (ATCC[®] CCL-
358 86[™]) were obtained from American Type Culture Collection. SK-BR3 was cultured in
359 complete DMEM with 10 % fetal bovine serum (FBS) and Raji was cultured in complete
360 RPMI 1640 with 10 % FBS. Human PBMCs were purified from anonymous healthy
361 volunteers using Histopaque density gradient centrifugation (Sigma-Aldrich). Human C1q
362 (CompTech) and hFcRn:hβ2m (Novus Biologicals) were purchased. All primers were
363 synthesized by Integrated DNA Technologies.

364 Preparation of recombinant proteins

365 Human Fc receptors including hFcγRI, hFcγRIIa, hFcγRIIb, hFcγRIIIa, and hFcRn:hβ2m
366 were cloned in-frame into the mammalian expression vector pcDNA3.4 using Gibson
367 Assembly cloning (NEB)⁴⁴ and were produced by transient transfection of Expi293 cells
368 (Invitrogen) according to the manufacturer's instruction^{43,44,65}. His-tagged Fc receptor
369 proteins were purified with Ni-NTA (GE Healthcare) affinity columns and GST-tagged Fc
370 receptor proteins were purified with Glutathione Sepharose (GE Healthcare) affinity columns
371 according to the manufacturer's instructions. In order to remove lipopolysaccharide (LPS)
372 and non-specifically bound protein, the Fc receptor-bound resins were washed with 50 mL of
373 PBS containing 0.1% Triton®X-114 (Sigma-Aldrich) and 50 mL of PBS. His-tagged Fc
374 receptor proteins were eluted with PBS containing 250 mM imidazole and GST-tagged Fc
375 receptor proteins were eluted with PBS containing 10 mM reduced L-glutathione. The buffer
376 in the Fc receptor protein containing eluent fraction was exchanged with PBS by Amicon
377 Ultra-30 centrifugal spin columns (Milipore).

378 Similarly, isolated Fc candidates were cloned in-frame into the mammalian expression
379 vector pcDNA3.4-IgH-Trastuzumab using Gibson Assembly cloning (NEB)⁶⁶ and were
380 produced in Expi293 cells (Invitrogen) grown for 5 days at 37°C with 8 % CO₂. All IgG
381 variants proteins purified by Protein A high capacity agarose resin (Thermo Scientific)
382 according to the manufacturer's instructions. Purity of antibody variants was confirmed by
383 SDS-PAGE and size exclusion chromatography and was over 95 %.

384 **Library Construction and screening**

385 Amino acid residues D249-R255, V308-L314, E430-Q438 in the Fc that are within 7Å
386 distance from FcRn in the crystal structure of the human Fc:FcRn complex (PDB ID:
387 4N0U)¹³ were selected for mutagenesis using the aa substitution scheme shown and three
388 separate libraries were constructed using primers with degenerate codons (**Supplementary**
389 **Table 1**). The heavy chain of trastuzumab IgG1 was used as the template. The amplified PCR
390 fragments were ligated into pPelB-AglycoT(H)-FLAG⁴³ with *SfiI* restriction sites for the
391 histidine scanning library. The resulting plasmids were transformed into *E. coli* Jude-1(F'
392 [Tn10(Tet^r) proAB⁺ lacI^q Δ(lacZ)M15] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) 80*dlacZ*ΔM15
393 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)7697 *galU galK rpsL endA1 nupG*) harboring
394 pBAD-AglycoT(L)-His^{43,67} to display both heavy and light chain of aglycosylated IgG1 in
395 periplasm anchored on the inner membrane via the NlpA signal peptide (APEX display)⁴⁵.
396 Following electroporation 1 × 10⁸ transformants were obtained. The transformants were
397 spheroplasted after overnight induction with 1mM of IPTG and 0.2% of L-arabinose,
398 followed by screening with 10 nM of hFcRn:hβ2m, conjugated with fluorescent Alexa488
399 (hFcRn:hβ2m Alexa488) as a target probe, at pH 5.8 in PBS. Screening was performed using
400 a FACSAria™ (BD Biosciences). In each round, the top 3 % of the population showing the
401 highest fluorescence was collected and the spheroplasts were re-sorted immediately to

402 remove false positives. The transformants with the plasmids of sorted spheroplasts were
403 prepared. After 4 rounds, in order to achieve pH-selective binding activity, the library was
404 first labeled with 5 nM of GST- hFcRn:h β 2m-Alexa647 at neutral pH, then incubated with 10
405 nM of hFcRn:h β 2m -Alexa488 at pH 5.8 (**Fig. 1a**). After the 5th round, single Fc variants
406 were analyzed with pH-dependent binding activities by flow cytometry and the four
407 candidates were isolated.

408 **LC-ESI-MS/MS analysis of Fc variants**

409 Fc proteins were analyzed by The University of Texas at Austin Proteomics Facility using
410 LC-MS on Thermo Orbitrap Fusion Tribrid mass spectrometer with the FT detector. A fast
411 gradient of 0.1% formic acid/water and 0.1% formic acid/acetonitrile over 10 minutes was
412 used to elute the intact proteins from an OPTI-TRAPTM protein microtrap (Optimize
413 Technologies). The Orbitrap Fusion was operated in Intact Protein Mode at 15,000
414 resolution from 400-2000 m/z. The data was deconvoluted using Thermo Protein
415 Deconvolution software.

416 **Size exclusion chromatography**

417 SEC analyses for the purified IgG proteins were performed on an ÄKTA Pure (GE
418 Healthcare) liquid chromatography system using a Superdex 200 10/300GC, (GE Healthcare),
419 with a mobile phase of pH 7.4 PBS at a flow rate of 0.75 mL/min. Chromatograms were
420 obtained by monitoring the absorbance at 280 nm. The injection amount was 100 μ g of
421 proteins in a volume of 200 μ L. For thermal stress test, each antibody protein (1 mg/ml) was
422 incubated at 70°C for 10, 20, 40 and 60 min and then analyzed by SEC using the above
423 described method.

424 **Differential scanning fluorimetry (DSF)**

425 DSF was performed with SYPRO Orange dye (Catalog #S-6650, Life Technologies) to
426 determine the thermal stability by monitoring the protein unfolding of the purified Fc
427 domains. For each sample, 15 μ L of Fc domains varying from 0.08 to 20 μ M were mixed
428 with 5 μ L of dye, diluted from 5000 \times to 20 \times for use. Each experiment included a control
429 without proteins containing 100 mM HEPES buffer at pH 7. The DSF assays were performed
430 on an Applied Biosystems ViiA 7 Real-Time PCR System using a temperature gradient from
431 25 $^{\circ}$ C to 99 $^{\circ}$ C at a temperature ramp rate of 0.03 $^{\circ}$ C/s. The fluorescence emissions and melting
432 temperature measurements were analyzed using ViiA 7 Software from Life Technologies.

433 **AC-SINS**

434 Goat anti-human Fc fragment specific (Jackson ImmunoResearch, 109-005-008) was buffer
435 exchanged into 20 mM potassium acetate (pH 4.3) twice using Zeba desalting columns
436 (Thermo Fisher Scientific, PI-89882). The goat anti-human Fc concentration was evaluated
437 using UV absorbance value at 280 nm (extinction coefficient of 1.26 mL/mg*cm). The
438 polyclonal antibody was diluted to 0.4 mg/mL in the acetate buffer. Equal volumes of goat
439 anti-human Fc antibody and gold nanoparticles (Ted Pella, 15705-1) were incubated at room
440 temperature (1 h). The antibody-gold conjugates (3 mL) were loaded onto 0.22 μ m filters
441 (Millipore Sigma, SLGV013SL, PVDF), resulting in the retention of the conjugates on the
442 filter membrane. PBS buffer (150 μ L) was added to the filter cartridges to elute the gold
443 conjugates, which typically resulted in recovery of \sim 100 μ L per cartridge. The concentrated
444 conjugates were mixed well by pipetting up and down ten times. The conjugates (5 μ L) were
445 added first to a 384-well polystyrene plate (Thermo Fisher, 12-565-506), and then the PBS
446 control or test antibodies (in PBS, 45 μ L) were added. The samples were mixed well by
447 pipetting up and down ten times. The absorbance spectra were measured (450-650 nm) in 1
448 nm increments using a Biotek Synergy Neo microplate reader (BioTek, Winooski VT). The

449 plasmon wavelengths were calculated by fitting a second order polynomial equations to the
450 40 absorbance measurements surrounding the maximum absorbance values and setting the
451 first derivatives of the polynomials to zero. The plasmon shifts were calculated as the
452 difference in the plasmon wavelengths of the test antibodies relative to the PBS control (no
453 human antibody).

454 **hFc:hFcRn KD determination using surface plasmon resonance (SPR)**

455 For SPR analysis, IgG1 antibody variants were immobilized on CM5 chip surface at a density
456 of ~500 response units (RU) via an amine coupling. hFcRn:h β 2m dimer (Novus Biologicals)
457 was injected at serially diluted concentrations (1000 nM – 40 nM) in PBS, pH 5.8, at a flow
458 rate of 30 μ L/min. To examine the interaction of hFcRn and antibody variants at
459 physiological pH 7.4, hFcRn:h β 2m dimer (Novus Biologicals) was immobilized on the
460 BIACore Chip at three different densities, 500 RU, 2000 RU, and 4000 RU via amine
461 coupling¹¹. Serially diluted antibodies (800 – 50 nM) were injected in PBS, pH 7.4, at a flow
462 rate of 30 μ L/min. Dissociation constants (K_{DS}) were determined by fitting the corresponding
463 binding isotherms for steady-state data or by fitting the kinetics for association and
464 dissociation employing a 1:1 Langmuir mass transfer model. In addition, to detect the
465 function of pH for hFc:hFcRn interaction, 800 nM of each antibody variants was injected
466 under diverse pH conditions (pH 6.0-8.0) and RU_{max} was measured.

467 **ELISA assays**

468 50 μ l of 2 μ g/ml antibody variant was diluted in PBS (pH 7.4) and used to coat 96 well
469 polystyrene ELISA plate (Corning, Corning, NY) overnight at 4°C. After blocking with PBS
470 (pH 7.4) with 3 % bovine serum albumin (BSA) for 1 hour at room temperature, the plate
471 was washed 4 times with PBS containing 0.05% Tween20 (PBST) and incubated with

472 serially diluted GST- hFcRn:hβ2m, His-tagged hFcγRI, GST-tagged hFcγRIIa, GST-tagged
473 hFcγRIIb, or GST-tagged hFcγRIIIa in PBS (pH 7.4) at room temperature for 1 hour. After
474 washing 4 times with PBST, either 1:10,000 diluted HRP conjugated anti-His or anti-GST
475 antibody (Rockland Immunochemicals) was added, and plates were washed with PBST.
476 Absorbance at 450 nm was measured after development with TMB substrate (Pierce
477 Biotechnology, Rockford, IL) according to the manufacturer's instructions.

478 **Rheumatoid factor (RF) binding assays**

479 50 μl of RF positive serum (Lee Biosolutions), obtained from three different RA patients,
480 was coated in 96 well polystyrene ELISA plate (Corning, NY) overnight at 4°C. After a
481 blocking and washing step, plates were incubated with biotinylated antibody variants for 1
482 hour at room temperature. The binding was detected by streptavidin-HRP and TMB substrate
483 (Thermo Fisher Scientific) according to the manufacturer's instructions.

484 **ADCC assays**

485 Her2-overexpressing SK-BR3 breast cancer cells were cultured in complete DMEM and
486 collected by centrifugation at 300× g for 5 min. The harvested SK-BR-3 cells were washed in
487 PBS and labeled with 4 μM Calcein AM (Life Technologies, USA) in PBS at 37°C under 5%
488 CO₂ for 30 min. The Calcein loaded SK-BR-3 cells were washed twice, resuspended in RPMI
489 medium, and seeded into a 96-well plate at 10,000 cells/well. The various concentrations of
490 IgG variants were also added. PBMCs were isolated from human blood from healthy donors.
491 Briefly, 50 mL of human blood was collected in heparinized vials (BD biosciences) and
492 mixed by gently inverting the tube several times. 25 mL of blood was layered over 25 mL of
493 Histopaque (Sigma-Aldrich) in 50 mL conical tubes. The tubes were centrifuged at 400 g for
494 10 min in a swing-out bucket with no brakes. The human PBMCs were aspirated in the

495 interphase between histopaque and medium. Human PBMCs were resuspended with red
496 blood cell (RBC) lysis buffer (155 mM NH₄Cl, 12 mM NaHCO₃, and 0.1 mM EDTA), and
497 washed twice with PBS. PBMCs were transferred into a 96-well plate at 100,000 cells/well
498 (the ratio of tumor versus PBMCs was 1:10), and the plates were incubated at 37°C under 5%
499 CO₂ for 4 hours. The released calcein AM was detected at excitation and emission
500 wavelengths of 485 nm and 535 nm. The percent of tumor cell lysis was calculated according
501 to the following formula; $(1) 100 \times (E-S) \times (M-S)^{-1}$, where E is the fluorescence of
502 experimental well, S is the fluorescence of a well containing tumor cells and complement but
503 no antibody, and M is the fluorescence of a well containing tumor cells with lysis buffer
504 (Triton X-100 at 2% v/v, SDS 1% w/v, 100mM NaCl, and 1mM EDTA). As an isotype
505 control, Rituximab was used.

506 **C1q deposition assay**

507 Raji cells were resuspended in 25% human serum and then incubated with the serially
508 diluted Rituximab-Fc variants at 37 °C for 30 min. Classical complement activation reactions
509 were then quenched with 20 volumes of ice-cold 1% BSA-PBS. The cells were pelleted,
510 washed once, and then probed with FITC anti-C1q (Abcam) for 30 min at 4 °C. The samples
511 were washed and analyzed by flow cytometry.

512 **CDC assay**

513 For CDC assay, Raji cells were labeled with 4 μM Calcein AM (Life Technologies, USA) in
514 PBS at 37°C under 5% CO₂ for 30 min and serially diluted Rituximab variants were
515 incubated with 25% human serum and calcein AM-loaded Raji cells at 37°C for 1 h in 96-
516 well plates. The supernatants were collected after centrifugation at 1,000g for 10 min. The

517 fraction of lysed Raji cells was determined by the same method as used for the ADCC assays.
518 As an isotype control, Trastuzumab was used.

519 **Mice**

520 Hemizygous hFcRn transgenic mice (line 276; aka Tg276) were generated by the F1 cross
521 of murine FcRn deficient B6.129X1-*Fcgrttm1Dcr/DcrJ* and hFcRn transgenic line (generated
522 by random integration of the hFcRn cDNA) B6.Cg-*Fcgrttm1Dcr* Tg (CAG-FCGRT) 276
523 Dcr/DcrJ (The Jackson laboratory)^{5,68}. Tg276 mice experiments were performed under a
524 protocol approved by UT Austin institutional Animal Care and Use Committee (IACUC).
525 hFcγR^{KI} mice⁵¹ were generated by Regeneron Pharmaceuticals Inc. to express hFcγRI,
526 hFcγRIIa_{H131}, hFcγRIIb_{I232}, hFcγRIIc_{stop13}, hFcγRIIIa_{V158} and hFcγRIIIb_{NA2} polymorphic
527 variants, as described previously⁵¹. hFcRn^{KI} (VG1481) and hβ2m^{KI} (VG5153) were designed
528 and generated by Regeneron Pharmaceuticals Inc. on a mixed 62.5% C57BL/6N and 37.5%
529 129S6/SvEv genetic background. hIgG1-heavy chain^{KI} (hIgG1^{KI}) were generated on a
530 BALB/c background in the Cogné lab. Human kappa-light chain^{KI} (*Igκ*^{KI}) were generated on
531 a C57BL/6N background in the Bruhns lab. Mice were intercrossed at Institut Pasteur
532 (Bruhns lab) to generate Marlene (hFcRn^{KI} hβ2m^{KI} hFcγR^{KI}) or Scarlett (hFcRn^{KI} hβ2m^{KI}
533 hFcγR^{KI} hIgG1,κ^{KI}) mice and used for experiments at 10 to 18 weeks of age. FcγR^{null} mice
534 expressing no FcγRs were used as controls. All mouse models demonstrated normal
535 development and breeding patterns and hFcγR expression on different cell populations from
536 the spleen were confirmed, as described previously⁵¹.

537 Experiments using knock in mice were validated by the Comité d'Éthique en
538 Expérimentation Animale (CETEA) #89 (Institut Pasteur, Paris, France) under #2013-0103
539 and by the French Ministry of Research under agreement #00513.02.

540 **Endogenous mouse or human IgG detection in knock-in mice**

541 Goat anti-human IgG-Fc fragment antibodies (1 μ g) or goat anti-mouse IgG-Fc fragment
542 antibodies (0.2 μ g) were diluted in carbonate buffer (pH 9.6) (Bethyl Laboratories,
543 Montgomery, TX) and used to coat MaxiSorp 96 well ELISA plates (Nunc, ThermoFischer
544 scientific, Waltham, MA) overnight at 4°C. After blocking with PBS with 3% BSA for 1
545 hour at room temperature, plates were washed 3 times with PBST and incubated with serially
546 diluted wt or KI mouse serum (or human and mouse reference serum as quantification
547 standards), at room temperature for 3 hours. After washing, HRP-conjugated anti-human IgG
548 (diluted 1:20,000, Bethyl Laboratories Inc.) or anti-mouse IgG (diluted 1:10,000, Bethyl
549 Laboratories Inc.) were added, and plates were washed with PBST. Absorbance at 492 vs 620
550 nm was measured after development with the OPD substrate (Sigma Aldrich, Saint-Louis,
551 MO).

552 **Immunohistochemistry**

553 Spleens were fixed in 10% neutral buffered formalin for 24h and embedded in paraffin.
554 Tissue sections (4 μ m) were blocked in PBS containing 3% BSA. Sections were stained with
555 either an anti-human FcRn antibody (4 μ g/mL, Novus Biologicals) or an anti-mouse FcRn
556 antibody (100 \times , R&D Systems) for 30 min at 37°C. Primary antibodies were detected with
557 appropriate peroxidase-conjugated secondary antibodies and DAB detection kit (<8min, RT).
558 All staining reactions were accompanied by a negative control that consisted of an isotype-
559 matched irrelevant antibody. Stained slides were evaluated with a light microscope (Nikon).

560 **RT PCR**

561 Total RNA was extracted from human peripheral blood mononuclear cells or murine
562 splenocytes using NucleoSpin RNA plus kit (Macherey-Nagel) according to the

563 manufacturer's recommendations. cDNAs were generated at 50 °C for 60 minutes using
564 random primers and SuperScript III Reverse Transcriptase (Invitrogen). The primer pairs for
565 the human FcRn gene *FCGRT* (L28-L179) and the mouse FcRn gene *Fcgrt* (L231-R295)
566 (*FCGRT*: 5'-CTCTCCCTCCTGTACCACCTT-3'; 5'-ATAGCAGGAAGGTGAGCTCCT-3';
567 *Fcgrt*: 5'-AGCTCAAGTCCGATTCTG-3'; 5'-GATCTGGCTGATGAATCTAGGTC-3')
568 were used for amplification with GoTaq G2 polymerase (Promega). Amplification was
569 performed by 35 cycles PCR each consisting of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1
570 min. At the end of the 35 cycles, samples were run for an additional 10 min at 72 °C and
571 analyzed by 1.5 % agarose gel electrophoresis.

572 **Pharmacokinetics studies in mice**

573 Animals were administered a bolus intravenous (i.v.) dose of 2 mg/kg antibody on day 0 by
574 tail vein injection. Blood samples were obtained from the tail vein using capillary pipettes at
575 different time points.

576 Serum concentration of antibody in Tg276, Marlene, or Scarlett was determined using a
577 quantitative ELISA as follows: 96-well plates were coated with 2 µg of Goat anti-human
578 F(ab')₂ fragment-specific F(ab')₂ (for Tg276, Jackson Immunoresearch) or 1 µg of each
579 antigens (for Marlene and Scarlett). Her2 extracellular domain protein (Antibodies-Online)
580 for trastuzumab, CD20 linear peptide (142-184, extracellular domain, Alpha Diagnostic Intl.,
581 Inc.) for rituximab, or IgE protein for omalizumab was used. Plates were blocked with 1%
582 Fish gelatin (AMRESCO Inc) in PBS for 1 hour, and then incubated with appropriately
583 diluted serum samples (1:200 for earlier time points and 1:50 or 1:100 for later time points).
584 Anti-kappa light chain antibody-HRP (Abcam) was used to detect the human antibody
585 (dilution 1:5,000). Absorbance at 450 nm was measured after development with TMB
586 substrate (Pierce Biotechnology, Rockford, IL) according to manufacturer's directions.

587 Standard curves were generated for each antibody variant diluted into 1:100 pre-bleed mouse
588 serum. The linear portions of standard curves generated in Prism (GraphPad Software) were
589 then used to quantify human IgG in the serum samples.

590 Pharmacokinetic parameters were analyzed with a two-compartment elimination model.
591 AUC_{inf} (area under the curve to infinity) was calculated using the log-linear trapezoidal
592 method²⁰. Terminal half-life ($T_{1/2}$) was calculated using two-phase clearance models. Log-
593 linear regression of the concentration data including at least the last six sampling time-points
594 with measurable concentrations. Serum clearance was estimated as: (2) $CL = Dose AUC_{inf}^{-1}$.
595 Volumes of distribution at steady state were calculated using the following equation: (3)
596 $V_{ss} = Dose \times AUMC_{inf} \times (AUC_{inf})^{-2}$.

597 **Computational Modeling**

598 All analysis was implemented in R and Stan, and can be found at [[https://github.com/meyer-](https://github.com/meyer-lab/FcRn-trafficking)
599 [lab/FcRn-trafficking](https://github.com/meyer-lab/FcRn-trafficking)], release 1.0 (doi: 10.5281/zenodo.3474026). Test conditions were
600 identified throughout to ensure model accuracy.

601 Exogenous IgG trafficking was modeled according to the following relationships,
602 consistent with the graphic presented in **Supplementary Fig. 10**. Exogenous IgG is modeled
603 to exchange between three compartments representing a central extracellular, peripheral
604 extracellular, and endosomal space. The central compartment is modeled as:

$$605 \quad (4) \frac{\delta C_c}{\delta t} = Q(C_p - C_c)$$

606 where C_c , C_p , and C_e indicate the central, peripheral, and endosomal compartment
607 concentrations, respectively, in units of $\mu\text{g/mL}$. Q indicates the transport rate between these
608 two compartments in units of the central compartment volume per hour. As the entire model
609 scales proportionally, the central compartment volume (V_c) was assumed to equal 1, and all

610 volumes are specified in relative amounts. The peripheral compartment concentration was
611 specified as:

$$612 \quad (5) \frac{\delta C_p}{\delta t} V_p = Q C_c - Q C_p - Q_u C_p + Q_u C_e f_{sort} f_{release}$$

613 where V_p and V_e are the peripheral and endosomal volumes (relative to the central
614 compartment volume), and Q_u is the rate of uptake into cells in units of central compartment
615 volume per hour.

616 f_{sort} indicates the fraction of endosomal IgG that is recycled, and $f_{release}$ indicates the
617 fraction of IgG presented to the cell surface that is released (as opposed to endocytosed
618 again).

$$619 \quad (6) \frac{\delta C_e}{\delta t} V_e = Q_u [C_p + C_e [(1 - f_{release}) f_{sort} - 1]]$$

620 Implicit in this model are a few assumptions: First, there is no clearance outside of cellular
621 uptake and lysosomal degradation. Each sorting fraction and model parameter is assumed to
622 not vary with the concentration of exogenous IgG, therefore assuming that the modeled
623 processes are not saturable. Finally, sorting and release are assumed to vary in the same order
624 as their measured affinities at pH 5.8 and 7.4, with IgG of no measurable affinity at 7.4 fully
625 released ($f_{release} = 1$).

626 The resultant linear ODE model was solved using the matrix exponential of the Jacobian. The half-life
627 was found through root finding with Newton's method. Model fitting was performed using Markov
628 Chain Monte Carlo within Stan⁶⁹. Priors on V_p , Q , and $V_i n$ were all specified as log-normal
629 distributions with a mean and deviation of 1 in their respective units. The prior for Q_u was specified
630 as a log-normal distribution with mean $e^{0.1}$ and deviation of 0.5. Sorting parameter priors were
631 specified to be flat and constrained to be in the order dictated by their relative affinity measurements.

632 For example, if species A had a higher endosomal affinity than B, then $f_{sort,A}$ was given an even prior
633 from 0 to 1, and $f_{sort,B}$ was given an even prior from 0 to $f_{sort,A}$. The half-life of each species was
634 compared to a normal distribution representing the average and standard error of the *in vivo*
635 experimental measurements. Sampling convergence was verified through autocorrelation analysis and
636 the Geweke criterion.

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648

649 **Author Contributions**

650 G.G., P.B., C.-H.L., and T.H.K. conceived and designed the research; C.-H.L., T.H.K., O.G.,
651 M.W., G.D., C.M.G., D.S., D.H., A.J.M., N.T., J.L. and E.M. performed experiments; G.G.,
652 P.B., C.-H.L., O.G., M.W., G.D., C.M.G., D.S., M.C., L.E.M., A.J.M., J.R.M., P.M.T., and
653 A.S.M. analyzed data. G.G., P.B., C.-H.L., M.W., and G.D. wrote the paper. G.G. and P.B.
654 provided funding.

655

656 **Competing Financial Interests**

657 G.G., C.-H.L., and T.H.K. declare competing financial interests (PCT/US2019/0048078). The
658 other authors declare no competing interests.

659

660 **Data availability**

661 All the other data that support the findings of this study are available from the corresponding
662 author upon request.

663 **Code availability**

664 All analysis for computational modeling was implemented in R v3.5.3 and rstan v2.18.2, and
665 can be found at [<https://github.com/meyer-lab/FcRn-trafficking>], release 1.1 (doi:
666 10.5281/zenodo.3474026). The code is available for access under the MIT license without
667 restrictions.

668

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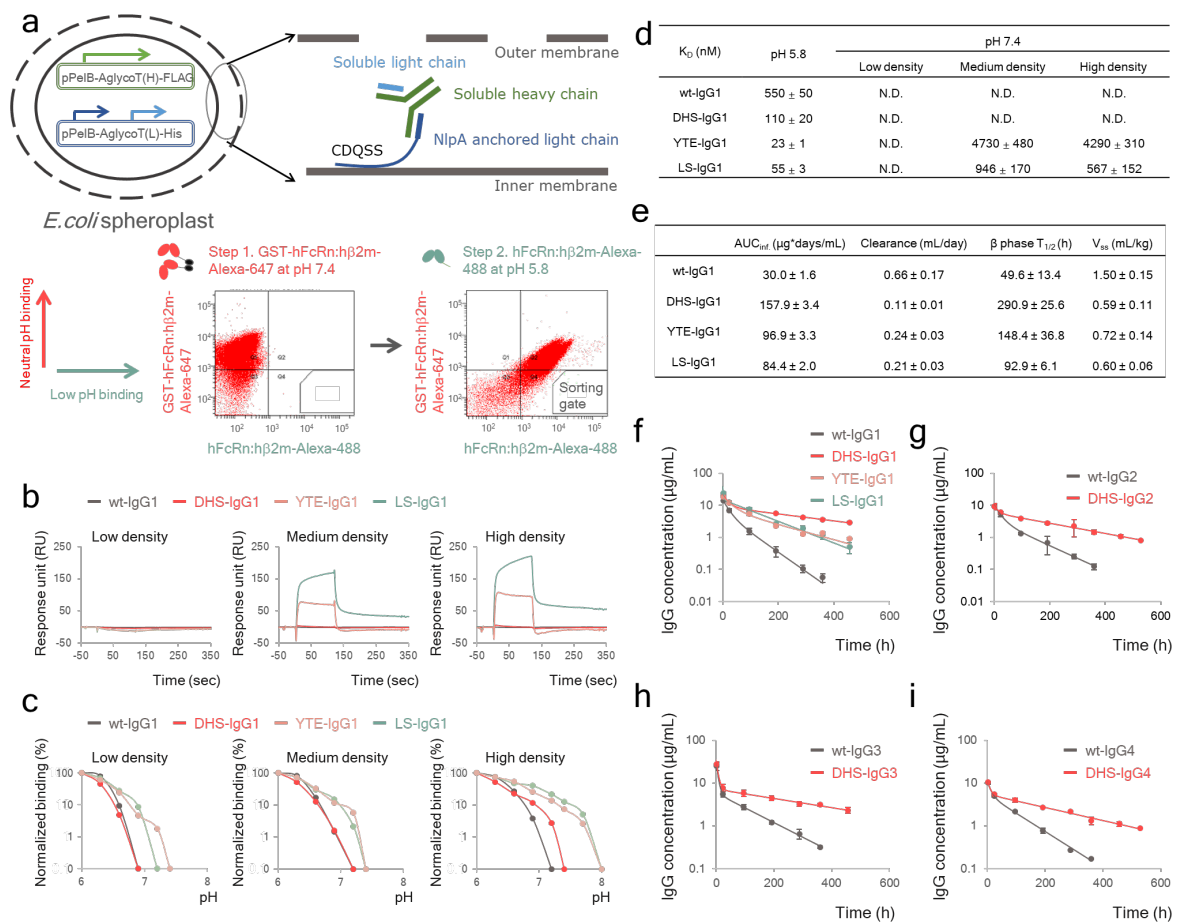
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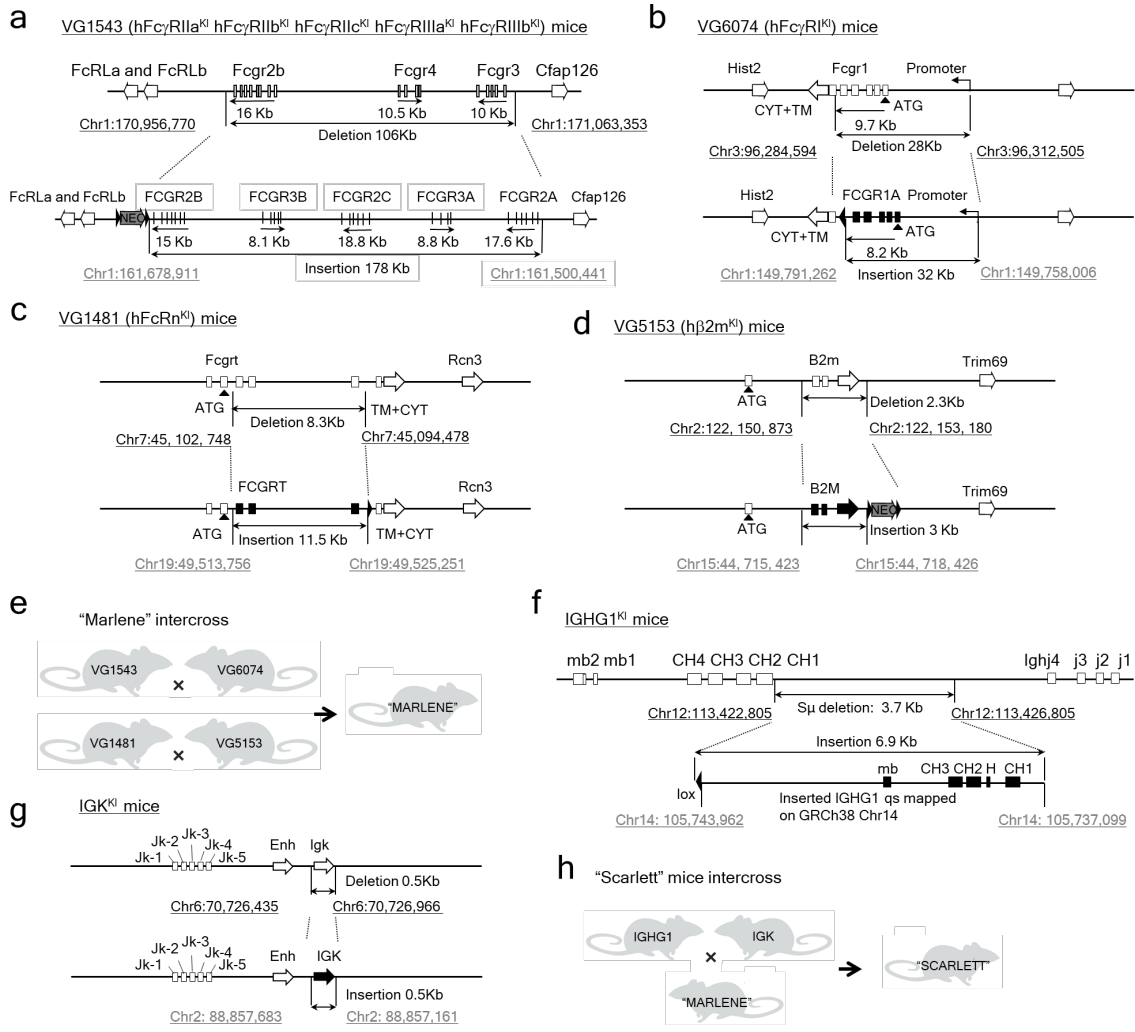
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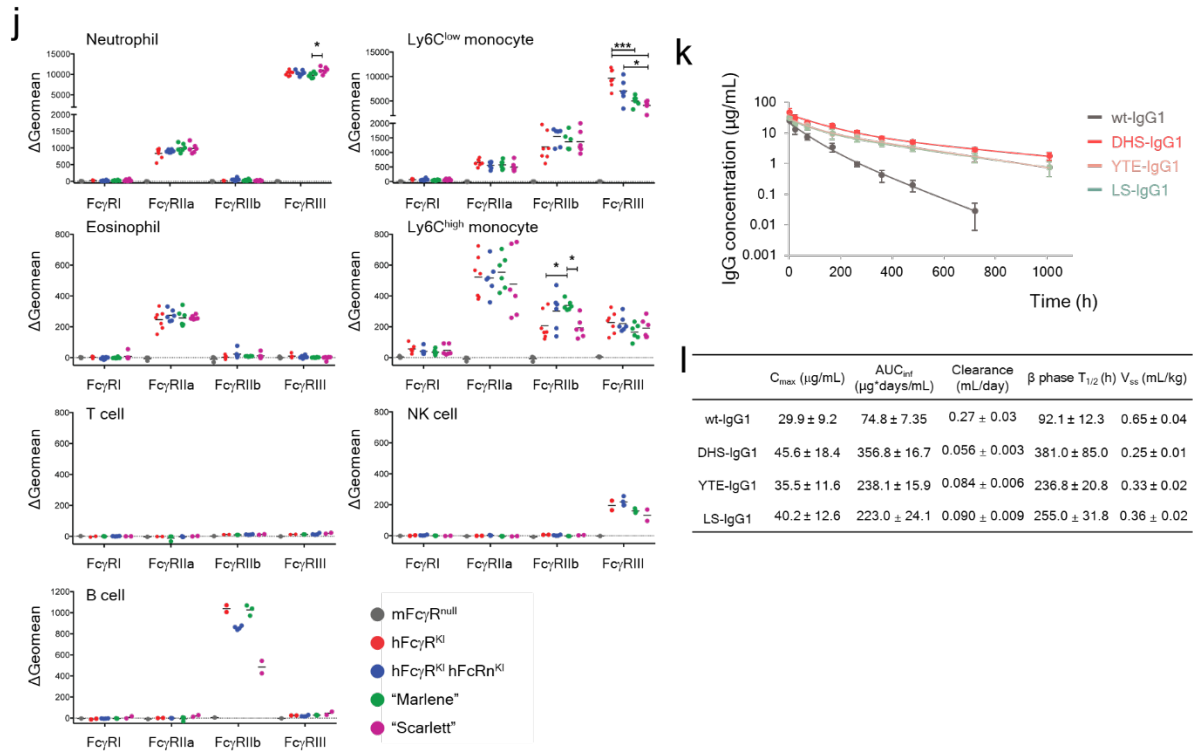


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838 **Figure 1. Engineering a human Fc domain with optimized pH-dependent FcRn binding for**
 839 **ultra-long circulation persistence.** (a) Screening strategy for the isolation of Fc mutations that
 840 confer favorable pH-dependent FcRn-binding using *E. coli* display. (b-c) SPR binding of IgG mutants
 841 (800 nM) to hFcRn:hp2m immobilized at low, medium or high density (500, 2000, and 4000 RU,
 842 respectively) either, (b) at pH 7.4 or (c) as a function of pH. Normalized binding intensity was
 843 calculated as the pH-dependent RU over the RU_{max} at pH 6.0, for antibodies at 800nM. Error bars:
 844 standard deviation from three independent experiments. (d-g) Serum antibody concentration of DHS
 845 formatted IgG1 (d), IgG2 (e), IgG3 (f) and IgG4 (g) antibodies in hemizygous Tg276 hFcRn
 846 transgenic mice as a function of time after administration. Each antibody variant (2 mg/kg) was
 847 administered intravenously to hemizygous Tg276 mice (n=11 for IgG1 and n=5 for IgG2, IgG3 and
 848 IgG4). Antibody concentrations were determined by ELISA. Data are presented as mean ± standard
 849 deviation.

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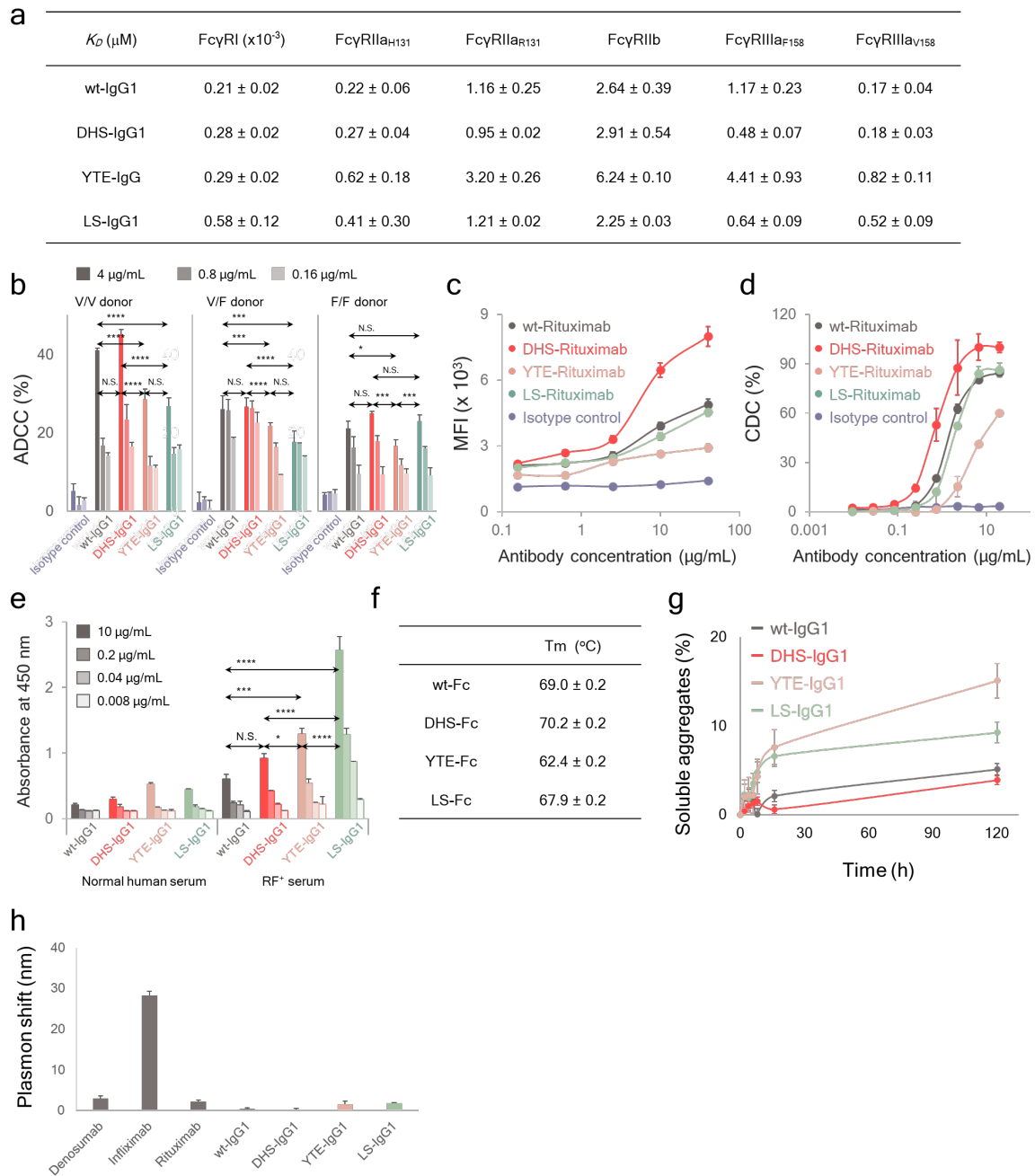
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Figure 2. IgG pharmacokinetics in new knock-in mouse models. (a-h) Generation of knock-in mice for human Fc γ Rs, FcRn, β 2m, IgG1 heavy chain and kappa light chain constant regions: (a) humanization of the mouse low-affinity receptor locus; (b) humanization of the ectodomains of mouse Fc γ RI; (c) humanization of the ectodomains of mouse FcRn; (d) humanization of the mouse β 2m gene (B2m); (e) breeding strategy to generate Marlene mice; (f) replacement of the switch μ region of the mouse heavy chain locus by the constant region of human IgG1 heavy chain (IGHG1); (g) humanization of the kappa light chain gene (IGK); (h) breeding strategy to generate Scarlett mice; (a-d, f-g) representations are not drawn to scale. Coordinates are based on mouse (GRCm38.p4) and human (GRCh38.p7) genomic assemblies; mouse genes are in empty rectangles, genomic coordinates are in black; human genes are in solid rectangles, genomic coordinates are in grey, black triangles represent LoxP sites. mb; exon encoding the transmembrane and intracytoplasmic domains; H; exon encoding the hinge domain. Neo; selection cassette. Mouse silhouette was created by co-author, Dr. Pierre Bruhns. (i) Immunohistochemical localization of hFcRn or mFcRn in tissue sections of human spleen, and spleen sections from of hFc γ R^{KI} mice (VG1543 \times VG6074) and Scarlett mice. Scale bar = 200 μm . (j) hFc γ R expression on different cell populations from the spleen of Fc γ R^{null} (negative control), hFc γ R^{KI} (positive control), hFcRn^{KI} hFc γ R^{KI}, Marlene and Scarlett mice. P-values by one-way Anova with Tukey's multiple comparison tests, * $P \leq 0.05$ and *** $P \leq 0.001$. (k) Change in serum IgG concentration following tail vein administration of 2 mg/kg of antibody in Scarlett (hFcRn^{KI} h β 2m^{KI} hFc γ R^{KI} hIgG1, κ ^{KI}) mice (n=6). Data are presented as mean \pm standard deviation.



873

874 **Figure 3. Features of DHS Fc variants relevant to therapeutic antibody development.** (a) ADCC
 875 assay of SK-BR-3 with PBMCs from Fc γ RIIIa *V/V*, *V/F*, or *F/F* donors. (b) C1q deposition on CD20⁺
 876 Raji cells revealed by flow cytometry. (c) CDC assay of Rituximab-Fc variants with Raji cells as a
 877 function of antibody concentration. (d) Binding to rheumatoid factor (RF) measured by ELISA. P-
 878 values by two-way ANOVA test, N.S. $P \geq 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq$
 879 0.0001. (e) Extent of antibody aggregation following thermal stress (50 °C incubation) of
 880 Trastuzumab variants, measured by size exclusion chromatography (SEC). (f) Antibody self-
 881 association properties measured by affinity-capture self-interaction nanoparticle spectroscopy (AC-

882 SINS) assay. Data are from one experiment representative of three experiments using three individual
883 donors (**a-d**). Errors in all plots and tables represent standard deviations from triplicate experiments.

884

885 **Table 1. K_D values for binding of DHS, YTE, LS variants or wt IgG1 to hFcRn:h β 2m dimer at**
 886 **pH 5.8 and 7.4.**

K_D (nM)	pH 5.8	pH 7.4		
		Low density	Medium density	High density
wt-IgG1	550 \pm 50	N.D.	N.D.	N.D.
DHS-IgG1	110 \pm 20	N.D.	N.D.	N.D.
YTE-IgG1	23 \pm 1	N.D.	4730 \pm 480	4290 \pm 310
LS-IgG1	55 \pm 3	N.D.	946 \pm 170	567 \pm 152

887 All IgG variants analyzed with hFcRn:h β 2m dimer and their kinetic values were analyzed by the
 888 equivalent binding model. Data are presented as mean \pm standard deviation from triplicate
 889 experiments.

890

891 **Table 2. Pharmacokinetic parameters for hemizygotic Tg276 hFcRn transgenic mice.**

	AUC _{inf} (μ g*days/mL)	Clearance (mL/day)	β phase $T_{1/2}$ (h)	V_{ss} (mL/kg)
wt-IgG1	30.0 \pm 1.6	0.66 \pm 0.17	49.6 \pm 13.4	1.50 \pm 0.15
DHS-IgG1	157.9 \pm 3.4	0.11 \pm 0.01	290.9 \pm 25.6	0.59 \pm 0.11
YTE-IgG1	96.9 \pm 3.3	0.24 \pm 0.03	148.4 \pm 36.8	0.72 \pm 0.14
LS-IgG1	84.4 \pm 2.0	0.21 \pm 0.03	92.9 \pm 6.1	0.60 \pm 0.06

892 Data are presented as mean \pm standard deviation (n=11).

893

894 **Table 3. Pharmacokinetic parameters for Scarlett (hFcRn^{KI} h β 2m^{KI} hFc γ R^{KI} hIgG1, κ ^{KI}) mice**

	C_{max} (mg/mL)	AUC _{inf} (μ g*days/mL)	Clearance (mL/day)	β phase $T_{1/2}$ (h)	V_{ss} (mL/kg)
wt-IgG1	29.9 \pm 9.2	74.8 \pm 7.35	0.27 \pm 0.03	92.1 \pm 12.3	0.65 \pm 0.04
DHS-IgG1	45.6 \pm 18.4	356.8 \pm 16.7	0.056 \pm 0.003	381.0 \pm 85.0	0.25 \pm 0.01
YTE-IgG1	35.5 \pm 11.6	238.1 \pm 15.9	0.084 \pm 0.006	236.8 \pm 20.8	0.33 \pm 0.02
LS-IgG1	40.2 \pm 12.6	223.0 \pm 24.1	0.090 \pm 0.009	255.0 \pm 31.8	0.36 \pm 0.02

895 Data are presented as mean \pm standard deviation (n=6).

896

897 **Table 4. K_D values for human FcγRs determined by SPR.**

K_D (μM)	FcγRI ($\times 10^{-3}$)	FcγRIIa _{H131}	FcγRIIa _{R131}	FcγRIIb	FcγRIIIa _{F158}	FcγRIIIa _{V158}
wt-IgG1	0.21 ± 0.02	0.22 ± 0.06	1.16 ± 0.25	2.64 ± 0.39	1.17 ± 0.23	0.17 ± 0.04
DHS-IgG1	0.28 ± 0.02	0.27 ± 0.04	0.95 ± 0.02	2.91 ± 0.54	0.48 ± 0.07	0.18 ± 0.03
YTE-IgG	0.29 ± 0.02	0.62 ± 0.18	3.20 ± 0.26	6.24 ± 0.10	4.41 ± 0.93	0.82 ± 0.11
LS-IgG1	0.58 ± 0.12	0.41 ± 0.30	1.21 ± 0.02	2.25 ± 0.03	0.64 ± 0.09	0.52 ± 0.09

898 All IgG variants analyzed with monomeric Fc receptors and their kinetic values were analyzed by the
899 equivalent binding model. Data are presented as mean ± standard deviation from triplicate
900 experiments.

901

902 **Table 5. T_m measurement by Differential Scanning Fluorimetry (DSF).**

	T _m (°C)
wt-Fc	69.0 ± 0.2
DHS-Fc	70.2 ± 0.2
YTE-Fc	62.4 ± 0.2
LS-Fc	67.9 ± 0.2

903 Data are presented as mean ± standard deviation (n=3).

904

905