



HAL
open science

IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies

Christian Leborgne, Elena Barbon, Jeffrey M Alexander, Hayley Hanby, Sandrine Delignat, Daniel M Cohen, Fanny Collaud, Saghana Muraleetharan, Dan Lupo, Joseph Silverberg, et al.

► To cite this version:

Christian Leborgne, Elena Barbon, Jeffrey M Alexander, Hayley Hanby, Sandrine Delignat, et al.. IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies. *Nature Medicine*, 2020, 26 (7), pp.1096-1101. 10.1038/s41591-020-0911-7 . hal-03031762

HAL Id: hal-03031762

<https://hal.science/hal-03031762v1>

Submitted on 11 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **IgG-cleaving endopeptidase enables *in vivo* gene therapy in the presence of anti-AAV**
2 **neutralizing antibodies**

3

4 Christian Leborgne^{1#}, Elena Barbon^{1#}, Jeffrey M. Alexander^{2#}, Hayley Hanby², Sandrine
5 Delignat³, Daniel M. Cohen², Fanny Collaud¹, Saghana Muraleetharan², Dan Lupo², Joseph
6 Silverberg², Karen Huang², Laetitia van Wittengerghe¹, Béatrice Marolleau¹, Adeline
7 Miranda¹, Anna Fabiano¹, Victoria Daventure³, Heena Beck², Xavier M. Anguela², Giuseppe
8 Ronzitti^{1#}, Sean M. Armour^{2#}, Sebastien Lacroix-Desmazes^{3*}, Federico Mingozzi^{1,2*}

9

10 ¹Integrare UMR-S951, Genethon, Inserm, Université d'Evry, Université Paris Saclay, 1 bis
11 rue de l'Internationale 91000 Evry, France

12 ²Spark Therapeutics, Inc. 3737 Market Street suite 1300, Philadelphia, PA 19104

13 ³Centre de recherche des Cordeliers, INSERM, Sorbonne Université, Université de Paris, F-
14 75006, Paris, France

15

16

17 [#]Contributed equally

18

19

20 ^{*}Corresponding authors:

21

22 Federico Mingozzi, PhD

23 Spark Therapeutics, Inc. 3737 Market Street suite 1300, Philadelphia, PA 19104, USA

24 Tel: +1 215 282 0134; Fax: +1 215 790 6248. e-mail: federico.mingozzi@sparktx.com

25

26 Sébastien Lacroix-Desmazes, PhD

27 INSERM UMR S 1138, Centre de Recherche des Cordeliers, Paris, F-75006 France

28 Tel: 01 44 27 81 93; Fax: 01 44 27 81 94. e-mail: sebastien.lacroix-desmazes@crc.jussieu.fr

29

69 **Abstract**

70

71 **Neutralizing antibodies (NAbs) to adeno-associated virus (AAV) vectors are highly**
72 **prevalent in humans^{1,2}, block liver transduction³⁻⁵ and vector readministration⁶, thus**
73 **representing a major limitation to *in vivo* gene therapy. Strategies aimed at overcoming**
74 **anti-AAV antibodies are being studied⁷, which often involve immunosuppression and**
75 **are not efficient in removing pre-existing antibodies. Imlifidase (IdeS) is an**
76 **endopeptidase able to degrade circulating IgG that is currently being tested in**
77 **transplant patients⁸. Here we studied if IdeS can eliminate anti-AAV antibodies in the**
78 **context of gene therapy. We showed efficient cleavage of pooled human IgG (IVIg) *in***
79 ***vitro* upon endopeptidase treatment. In mice passively immunized with IVIg, IdeS**
80 **administration decreased anti-AAV antibodies and enabled efficient liver gene transfer.**
81 **The approach was scaled up to non-human primates, a natural host for wild type AAV.**
82 **IdeS treatment prior to AAV vector infusion was safe and resulted in enhanced liver**
83 **transduction, even in the setting of vector readministration. Finally, IdeS reduced anti-**
84 **AAV antibody levels from human plasma samples *in vitro*, including plasma from**
85 **prospective gene therapy trial participants. These results provide a potential solution to**
86 **overcome pre-existing antibodies to AAV-based gene therapy.**

87

88

89 **Main**

90

91 Gene therapy can treat a variety of human diseases through systemic administration of adeno-
92 associated virus (AAV) vectors⁹⁻¹². Yet, pre-treatment neutralizing antibodies (NAbs) directed
93 against the AAV capsid pose a major limitation to AAV gene transfer, as they block target
94 tissue transduction even at relatively low titers³⁻⁵.

95 Here, we explored the use of the IgG-degrading enzyme derived from *Streptococcus pyogenes*
96 Imlifidase (IdeS)¹³ as a strategy to overcome the limitation of NAbs to AAV. IdeS is an
97 endopeptidase that cleaves human IgG into F(ab')₂ and Fc fragments, which was successfully
98 tested in clinical trials in the setting of antibody-mediated graft rejection⁸ and preclinically
99 heparin-induced thrombocytopenia¹⁴. Commercially sourced (IdeS-C) or lab-made (IdeS)
100 endopeptidases showed similar cleavage activity of purified human IgG (intravenous
101 immunoglobulin, IVIg) *in vitro*, resulting in a complete degradation of IgG into F(ab')₂ and
102 Fc at 24 hours (Fig. 1a) and a significant decrease in anti-AAV serotype 8 (AAV8) IgG
103 concentration (Fig. 1b), and NAb titer¹⁵ (Extended Data Fig. 1a). No effect of IdeS on AAV8
104 transduction of HEK293 cells *in vitro* was noted (Extended Data Fig. 1b). Because IdeS does
105 not cleave efficiently mouse IgG¹⁶, a first *in vivo* experiment was performed using a passive
106 immunization model of gene transfer¹⁷, in which C57BL/6 mice were infused with
107 neutralizing amounts of IVIg and then treated with IdeS prior to the administration of an
108 AAV8 vector expressing Gaussia luciferase (AAV8-GLuc, Fig. 1c). Consistent with human
109 studies^{18,19}, IdeS was cleared from the circulation within 24-48 hours from the infusion in
110 mice (Fig. 1d) and non-human primates (Extended Data Fig. 1c). Cleavage of IgG resulted in
111 detection of residual levels of single-chain IgG (scIgG) along with F(ab')₂ and Fc fragments
112 (Fig. 1d). Anti-AAV8 IgG and NAb titers were significantly decreased in IdeS-treated
113 animals (IVIg/IdeS cohort, Fig. 1e,f) and, accordingly, GLuc expression levels were in the

114 range of those of control animals receiving PBS followed by IdeS (PBS/IdeS cohort, Fig. 1g).
115 Conversely, animals treated with IVIg followed by PBS showed no transgene expression
116 (IVIg/PBS cohort, Fig. 1g). Vector genomes in liver were undetectable in IVIg/PBS animals
117 (Extended Data Fig. 1d). IVIg/IdeS-treated mice showed a partially decreased vector genome
118 copy number (VGCN) compared to PBS/IdeS controls (Extended Data Fig. 1d), likely due to
119 some degree of vector neutralization mediated by residual circulating IgG fragments which
120 were not detected in the NAb assay (Fig. 1d). The same experimental setting was also applied
121 to a therapeutically relevant mouse model of hemophilia B (HB), in which animals received
122 an AAV8 vector expressing human coagulation factor IX (hFIX) following passive
123 immunization with IVIg (Fig. 1c). In this setting, treatment with IdeS also resulted in
124 elimination of anti-AAV8 IgG and NAb (Extended Data Fig. 1e,f), with complete rescue of
125 transgene expression (Fig. 1h) and liver transduction (Extended Data Fig. 1g), and correction
126 of the bleeding diathesis (Fig. 1i).

127 We next tested IdeS in non-human primates (NHPs, *Macaca fascicularis*). NHPs are natural
128 hosts for AAV²⁰, and therefore carry anti-AAV NAb. Two male animals, NHP3 and NHP5
129 with similar anti-AAV8 NAb titers (1:17.2, Supplementary Table 1), were infused with
130 2×10^{12} vector genomes (vg)/kg of an AAV8-hFIX vector. Prior to vector infusion, NHP5
131 received two infusions of IdeS (Fig. 2a). Following IdeS administration, total circulating IgG
132 in NHP5 decreased significantly (Fig. 2b). IgG cleavage was confirmed by Western blot,
133 although a larger proportion of IgG and sIgG remained detectable (Fig. 2c) compared to
134 human IgG in mouse plasma (Fig. 1d). Despite the partial cleavage, anti-AAV8 IgG and NAb
135 titers in NHP5 decreased following IdeS treatment, while they remained unchanged in the
136 control animal NHP3 (Fig. 2d,e, horizontal dashed line). Accordingly, following vector
137 administration, hFIX transgene expression peaked and plateaued³ at significantly higher hFIX
138 levels in NHP5 compared to the control animal NHP3 (Fig. 2f). Consistently, VGCN analysis

139 in liver at sacrifice showed higher transduction levels in NHP5 compared to NHP3 (Fig. 2g).
140 The presence of anti-IdeS antibodies before treatment in NHP5 (Supplementary Table 1) did
141 not prevent rescue of liver transduction with IdeS. After vector administration, the IdeS-
142 treated animal NHP5 developed lower anti-AAV8 IgG and NAb titers than the control animal
143 NHP3 (Fig. 2h,i).

144 Two additional NHPs with anti-AAV8 NAb titers of 1:3.16 were then studied (NHP2 and
145 NHP6, Supplementary Table 1), to test the efficacy of IdeS at lower pre-existing antibody
146 titers. IdeS treatment resulted in digestion of total IgGs (Extended Data Fig. 2a), reduction of
147 anti-AAV8 IgG and NABs (Extended Data Fig. 2b,c), and enhanced transgene expression and
148 liver transduction in NHP6 (Extended Data Fig. 2d,e). No change in anti-AAV8 IgG and
149 NABs was observed in the control animal NHP2 (Extended Data Fig. 2b,c, horizontal dashed
150 line), which had lower transgene expression and VGCN than NHP6 (Extended Data Fig.
151 2d,e). Following vector administration, both animals had an increase in anti-AAV8 IgG
152 (Extended Data Fig. 2f) and NAb titers (Extended Data Fig. 2g), although in NHP6, the IdeS-
153 treated animal, the increase was mostly transient. Administration of vector alone or in
154 combination with IdeS was not associated with evidence of toxicities (Supplementary Table
155 2). Together, these results indicate that IdeS treatment reduces natural humoral immunity to
156 AAV and enhances AAV vector transduction upon systemic delivery in NHPs.

157

158 We next tested the IdeS technology in the context of AAV vector re-administration. A pilot
159 study was conducted in two animals, NHP1 and NHP4 (Extended Data Fig. 3a), with pre-
160 existing NABs to AAV8 (1:31.6, Supplementary Table 1). NHP4 received IdeS prior to vector
161 administration, resulting in a significant decrease of anti-AAV8 IgG (Extended Data Fig. 3b).
162 No change in anti-AAV8 IgG was observed in control animal NHP1 (Extended Data Fig. 3b,
163 horizontal dashed line). Following the first vector administration, the IdeS-treated animal,

164 NHP4, showed higher circulating hFIX levels compared to the control animal, NHP1
165 (Extended Data Fig. 3c). Both animals developed anti-AAV8 IgG and NAbs following vector
166 infusion, although the IdeS-pretreated animal, NHP4, developed significantly lower anti-
167 AAV8 IgG (Extended Data Fig. 3d). At day 82 post-vector infusion, both animals received
168 two doses of IdeS (500 µg/kg, one day apart, Extended Data Fig. 3a). In both animals, IdeS
169 treatment resulted in the digestion of circulating IgG, although large amounts of scIgG and, to
170 a lesser extent, IgG remained detectable (Extended Data Fig. 3e). Accordingly, anti-AAV8
171 IgG as well as NAbs titers decreased in both animals (Extended Data Fig. 3f,g). Following the
172 second vector administration, an increase in hFIX circulating levels was observed in NHP4
173 (Extended Data Fig. 4a). NHP1 developed anti-human FIX antibodies prior to vector re-
174 administration (Extended Data Fig. 4b and Supplementary Table 1), a phenomenon
175 commonly seen in NHPs^{21,22}, thus preventing the measurement of hFIX circulating levels.
176 Administration of IdeS in NHP1 resulted in a transient decrease in anti-hFIX antibody levels
177 (Extended Data Fig. 4b), which did not result in detection of circulating hFIX (Extended Data
178 Fig. 4a). VGCN measured in liver showed a higher transduction of hepatocytes in NHP4
179 compared to NHP1 (Extended Data Fig. 4c), reflecting the fact that NHP1 had residual
180 antibodies to AAV8 at the time of both the first and the second vector infusions (Extended
181 Data Fig. 3b,f,g). Following vector re-administration, NHP4, the animal that received IdeS at
182 days -1, 82 and 83 (Extended Data Fig. 3a), did not develop anti-AAV8 IgG (Extended Data
183 Fig. 4d) or NAbs (Extended Data Fig. 4e), and had a lower IgM response compared to NHP1
184 (Extended Data Fig. 4f).

185 A study in a large cohort of NHPs (*Chlorocebus sabaeus*) was then conducted. Eight animals
186 were immunized with an AAV vector serotype AAV-LK03²³ at a dose of 2×10^{12} vg/kg at day
187 0, and then at day 210 randomly assigned to a PBS control group (n=3) or a IdeS treatment
188 group (n=5) (Fig. 3a). Vector administration at day 0 resulted in development of a broad

189 range of anti-AAV-LK03 IgG and NAb titers (Fig. 3b-e). A single IdeS treatment resulted in
190 a decrease of anti-AAV-LK03 IgG (Fig. 3b,c) and NAb (Fig. 3d,e) titers in all animals, while
191 no significant change in titers was observed in the PBS treatment group. Accordingly,
192 administration of an AAV-LK03 vector expressing human factor VIII (AAV-LK03-hFVIII)
193 on day 211 resulted in significantly higher expression of the hFVIII transgene in the IdeS
194 treated animals compared to controls (Fig. 3f-h). Following the second vector infusion, all
195 animals developed anti-capsid IgM (Extended Data Fig. 5a,b) and IgG (Extended Data Fig.
196 5c,d). As previously described²⁴, hFVIII expression was transiently detected because of the
197 formation of antibodies to hFVIII (Extended Data Fig. 5e,f).

198 Analysis of aggregated data from 6 NHPs, NHP2 and NHP3 having received also a double
199 IdeS injection at day 35, showed, as observed in humans⁸, a good safety profile
200 (Supplementary Tables 2-5) and a transient degradation of total IgG in all animals after IdeS
201 administration (Extended Data Fig. 6a). All animals dosed with IdeS developed binding
202 antibodies to the enzyme after treatment (Extended Data Fig. 6b).

203 Next, to study the effect of IdeS on anti-AAV IgG in humans, we treated plasma from healthy
204 donors and from a cohort of Crigler-Najjar (CN) patients²⁵, a rare liver disease for which an
205 AAV gene therapy trial is ongoing (NCT03466463). Following *in vitro* incubation with IdeS,
206 anti-AAV8 IgG titers were significantly decreased in all subjects tested (Fig. 4a). The
207 presence of anti-IdeS antibodies was then evaluated in sera from 52 healthy donors (Extended
208 Data Fig. 6c), confirming the previously reported seroprevalence data¹⁸. Anti-IdeS antibodies
209 did not affect the cleavage of IgG specific to the enzyme (Fig. 4b), although a less efficient
210 degradation of IgG was observed in serum from NHPs after immunization against IdeS
211 (Extended Data Fig. 7a-d). Finally, to further bridge the results obtained in NHPs to humans,
212 Western blot analysis of IgG after IdeS digestion *in vitro* was performed. Complete cleavage
213 of human IgG was shown, while residual levels of scIgG, which can retain partial

214 neutralization activity, were found only in NHPs (Fig. 4c). Combined with the *in vitro* IgG
215 digestion data (Fig. 4a) and published results in humans⁸, these results suggest that IdeS might
216 more efficiently remove anti-AAV NAbs in humans compared to NHPs.

217

218 Anti-capsid antibodies are a major limitation to AAV vector-mediated gene transfer. Several
219 strategies have been attempted to circumvent anti-AAV NAbs⁶, although to date seropositive
220 individuals are mostly declared ineligible to receive AAV-based treatments when the vector is
221 administered systemically. Here we showed that a one-time *in vivo* administration of IdeS
222 results in a decrease in anti-AAV antibody titers and allows the successful transduction of the
223 liver, in the setting of both pre-existing natural immunity to AAV and, possibly, vector
224 readministration. The procedure appeared to be safe, consistent with studies in healthy
225 individuals¹⁸ and in patients undergoing graft transplantation⁸, which showed that transient
226 digestion of circulating IgG was not associated with severe adverse events and did not appear
227 to increase the risk of opportunistic infections.

228 Natural humoral immunity to IdeS, deriving from exposure to *Streptococcus pyogenes*, may
229 represent a limitation to the use of the technology. While this is a potential concern, our
230 results would argue that IdeS is active even in the presence of antibodies against the enzyme
231 itself. Repeated administrations of IdeS can eventually result in neutralizing antibodies to the
232 enzyme, although it has been shown that humoral immune responses triggered by IdeS in
233 humans are only transient¹⁸.

234

235 In summary, results presented here indicate that IdeS can efficiently cleave anti-AAV
236 antibodies thereby enhancing IgG clearance and reducing their AAV neutralizing activity in
237 blood to levels compatible with transduction of hepatocytes. Pre-treatment with IdeS at the
238 time of AAV vector infusion is a potential strategy to enable efficient systemic gene transfer

239 in the presence of low to moderate pre-existing anti-capsid antibodies, which are commonly
240 found in humans¹⁷, and will possibly allow for repeated vector administration. This work
241 provides key proof-of-concept of data on the use of IdeS technology to address a major
242 limitation of *in vivo* gene transfer with AAV vectors. Future studies will help translating these
243 findings to human trials.
244

245 **Acknowledgments**

246

247 We thank the staff of ONIRIS and Virscio staff for their help in designing and performing the
248 NHP studies. We also thank the Spark Therapeutics vector production, bioanalytical,
249 immunology, liver, sample management, and laboratory information management teams for
250 their contribution to the NHP studies. The work was supported by Institut National de la Santé
251 et de la Recherche Médicale, Centre National de la Recherche Scientifique, Sorbonne
252 Université.

253

254 **Author contributions**

255

256 C.L., E.B., J.M.A, S.D., H.H., F.C., S.M., D.L., J.S., K.H., L.v.W., B.M., A.M., A.F., V.D.,
257 D.M.C., and H.B. performed the experiments. C.L., G.R., S.M.A., S.L.-D., and F.M. analyzed
258 the results and wrote the manuscript. X.M.A., S.M.A., S.L.-D., and F.M. conceived and
259 directed the studies.

260

261 **Competing interests**

262

263 The authors declare the following competing interests.

264 **Employment.** J.M.A., H.H., S.M., D.L., J.S., K.H., D.M.C., H.B., X.M.A., S.M.A., and F.M.
265 are employees of Spark Therapeutics, Inc. a Roche company.

266 **Patent.** F.M., S.L.D., S.M.A. and C.L. are inventors on a patent related to this work. Patent
267 applicants: INSERM (Institut National de la Sante et de la Recherche Medicale), Genethon,
268 Sorbonne Universite, Universite Paris Descartes, Universite Paris Diderot, Spark
269 Therapeutics, Inc; inventors: Sebastien Lacroix-Desmazes, Federico Mingozzi, Christian

270 Leborgne, Jordan D Dimitrov, Sean M. Armour; application number: PCT/EP2019/069280;
271 application request filed. Specific aspects of manuscript covered in the patent application: use
272 of IdeS to decrease pre-existing antibody immunity to AAV; use of IdeS to allow for vector
273 readministration.

274

275 **References main text**

276

- 277 1 Boutin, S. *et al.* Prevalence of serum IgG and neutralizing factors against adeno-
278 associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population:
279 implications for gene therapy using AAV vectors. *Hum Gene Ther* **21**, 704-712,
280 doi:10.1089/hum.2009.182 (2010).
- 281 2 Li, C. *et al.* Neutralizing antibodies against adeno-associated virus examined
282 prospectively in pediatric patients with hemophilia. *Gene Ther*, doi:gt201190 [pii]
283 10.1038/gt.2011.90 (2011).
- 284 3 Jiang, H. *et al.* Effects of transient immunosuppression on adenoassociated, virus-
285 mediated, liver-directed gene transfer in rhesus macaques and implications for human
286 gene therapy. *Blood* **108**, 3321-3328, doi:10.1182/blood-2006-04-017913 [pii] 10.1182/blood-
287 2006-04-017913 (2006).
- 288 4 Manno, C. S. *et al.* Successful transduction of liver in hemophilia by AAV-Factor IX
289 and limitations imposed by the host immune response. *Nat Med* **12**, 342-347,
290 doi:10.1038/nm1358 [pii] 10.1038/nm1358 (2006).
- 291 5 Scallan, C. D. *et al.* Human immunoglobulin inhibits liver transduction by AAV
292 vectors at low AAV2 neutralizing titers in SCID mice. *Blood* **107**, 1810-1817,
293 doi:10.1182/blood-2005-08-3229 [pii] 10.1182/blood-2005-08-3229 (2006).
- 294 6 Mingozzi, F. & High, K. A. Overcoming the Host Immune Response to Adeno-
295 Associated Virus Gene Delivery Vectors: The Race Between Clearance, Tolerance,
296 Neutralization, and Escape. *Annu Rev Virol* **4**, 511-534, doi:10.1146/annurev-
297 virology-101416-041936 (2017).

- 298 7 Meliani, A. *et al.* Antigen-selective modulation of AAV immunogenicity with
299 tolerogenic rapamycin nanoparticles enables successful vector re-administration. *Nat*
300 *Commun* **9**, 4098, doi:10.1038/s41467-018-06621-3 (2018).
- 301 8 Jordan, S. C. *et al.* IgG Endopeptidase in Highly Sensitized Patients Undergoing
302 Transplantation. *N Engl J Med* **377**, 442-453, doi:10.1056/NEJMoa1612567 (2017).
- 303 9 George, L. A. *et al.* Hemophilia B Gene Therapy with a High-Specific-Activity Factor
304 IX Variant. *N Engl J Med* **377**, 2215-2227, doi:10.1056/NEJMoa1708538 (2017).
- 305 10 Mendell, J. R. *et al.* Single-Dose Gene-Replacement Therapy for Spinal Muscular
306 Atrophy. *N Engl J Med* **377**, 1713-1722, doi:10.1056/NEJMoa1706198 (2017).
- 307 11 Miesbach, W. *et al.* Gene therapy with adeno-associated virus vector 5-human factor
308 IX in adults with hemophilia B. *Blood*, doi:10.1182/blood-2017-09-804419 (2017).
- 309 12 Rangarajan, S. *et al.* AAV5-Factor VIII Gene Transfer in Severe Hemophilia A. *N*
310 *Engl J Med* **377**, 2519-2530, doi:10.1056/NEJMoa1708483 (2017).
- 311 13 von Pawel-Rammingen, U., Johansson, B. P. & Bjorck, L. IdeS, a novel streptococcal
312 cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J* **21**, 1607-
313 1615, doi:10.1093/emboj/21.7.1607 (2002).
- 314 14 Kizlik-Masson, C. *et al.* Cleavage of anti-PF4/heparin IgG by a bacterial protease and
315 potential benefit in heparin-induced thrombocytopenia. *Blood* **133**, 2427-2435,
316 doi:10.1182/blood.2019000437 (2019).
- 317 15 Meliani, A. *et al.* Determination of anti-adeno-associated virus vector neutralizing
318 antibody titer with an in vitro reporter system. *Hum Gene Ther Methods*,
319 doi:10.1089/hgtb.2015.037 (2015).
- 320 16 Agniswamy, J., Lei, B., Musser, J. M. & Sun, P. D. Insight of host immune evasion
321 mediated by two variants of group a Streptococcus Mac protein. *J Biol Chem* **279**,
322 52789-52796, doi:10.1074/jbc.M410698200 (2004).

- 323 17 Mingozi, F. *et al.* Overcoming preexisting humoral immunity to AAV using capsid
324 decoys. *Sci Transl Med* **5**, 194ra192, doi:10.1126/scitranslmed.3005795 (2013).
- 325 18 Winstedt, L. *et al.* Complete Removal of Extracellular IgG Antibodies in a
326 Randomized Dose-Escalation Phase I Study with the Bacterial Enzyme IdeS--A Novel
327 Therapeutic Opportunity. *PLoS One* **10**, e0132011, doi:10.1371/journal.pone.0132011
328 (2015).
- 329 19 Lorant, T. *et al.* Safety, immunogenicity, pharmacokinetics, and efficacy of
330 degradation of anti-HLA antibodies by IdeS (imlifidase) in chronic kidney disease
331 patients. *Am J Transplant* **18**, 2752-2762, doi:10.1111/ajt.14733 (2018).
- 332 20 Gao, G. P. *et al.* Novel adeno-associated viruses from rhesus monkeys as vectors for
333 human gene therapy. *Proc Natl Acad Sci U S A* **99**, 11854-11859,
334 doi:10.1073/pnas.182412299 182412299 [pii] (2002).
- 335 21 Mingozi, F. *et al.* Pharmacological modulation of humoral immunity in a nonhuman
336 primate model of AAV gene transfer for hemophilia B. *Mol Ther* **20**, 1410-1416,
337 doi:mt201284 [pii] 10.1038/mt.2012.84 (2012).
- 338 22 Nathwani, A. C. *et al.* Long-term safety and efficacy following systemic
339 administration of a self-complementary AAV vector encoding human FIX
340 pseudotyped with serotype 5 and 8 capsid proteins. *Mol Ther* **19**, 876-885,
341 doi:mt2010274 [pii] 10.1038/mt.2010.274 (2011).
- 342 23 Lisowski, L. *et al.* Selection and evaluation of clinically relevant AAV variants in a
343 xenograft liver model. *Nature* **506**, 382-386, doi:10.1038/nature12875 (2014).
- 344 24 McIntosh, J. *et al.* Therapeutic levels of FVIII following a single peripheral vein
345 administration of rAAV vector encoding a novel human factor VIII variant. *Blood*,
346 doi:blood-2012-10-462200 [pii] 10.1182/blood-2012-10-462200 (2013).

347 25 Aronson, S. J. *et al.* Prevalence and Relevance of Pre-Existing Anti-Adeno-Associated
348 Virus Immunity in the Context of Gene Therapy for Crigler-Najjar Syndrome. *Hum*
349 *Gene Ther* **30**, 1297-1305, doi:10.1089/hum.2019.143 (2019).

350

351

352 **Figures legends**

353

354 **Figure 1** IdeS degrades anti-AAV antibodies and allows for successful liver transduction in
355 mice passively immunized with IVIg. **a**, Western blot analysis of IVIg incubated 5 minutes
356 and 24 hours with commercial (IdeS-C) or lab-made (IdeS) endopeptidase, or with PBS. The
357 predicted molecular weight of intact IgG, single-chain IgG (scIgG), F(ab')₂ and Fc fragments
358 is shown. One representative out of two independent experiments shown. **b**, Anti-AAV8 IgG
359 concentration in IVIg measured after a 24-hour incubation with PBS, IdeS-C, or IdeS (n=1
360 per condition tested in duplicate, one representative out of two independent experiments
361 shown). **c**, Protocol outline. C57BL/6 (n=6 mice per group, one representative out of two
362 independent experiments shown) or hemophilia B (HB, n=4 mice per group, one experiment)
363 mice were passively immunized with IVIg or received PBS as control. Thirty minutes later,
364 mice were injected with either IdeS or PBS one day prior to the delivery of AAV8 -GLuc or -
365 hFIX vectors. **d**, Western blot analysis of human IgG in serum of passively immunized
366 C57BL/6 mice before or 30 minutes and 24 hours after IdeS treatment. One representative out
367 of two independent experiments shown. **e,f**, Effect of IdeS on **e**, anti-AAV8 IgG and **f**, NAb
368 titers measured 24 hours after treatment. Bars represent the group mean ± s.d. (n=6 mice per
369 group). **g**, GLuc activity in serum of passively immunized mice treated with IdeS (IVIg/IdeS)
370 or PBS (IVIg/PBS). Naïve mice receiving IdeS were used as controls (PBS/IdeS). RLU,
371 Relative Light Units. **h,i**, Rescue of transgene expression in passively immunized HB mice
372 (n=4) treated with IdeS (IVIg/IdeS) or PBS (IVIg/PBS) prior to AAV8-hFIX delivery. Naïve
373 HB mice receiving IdeS were used as controls (PBS/IdeS). **h**, hFIX in plasma measured by
374 ELISA over time. **i**, bleeding time following tail clip assay. All data are shown as mean ± s.d.
375 Statistical analyses were performed by **b**, repeated measures one-way ANOVA with
376 Dunnett's multiple comparisons test; **e**, one-way ANOVA with Tukey's multiple comparisons

377 test; **f**, one-way ANOVA, non-parametric with Dunn's multiple comparisons test; **g**, two-way
378 ANOVA with Dunnett's multiple comparisons test (vs. IVIg/IdeS and PBS/IdeS); **h**, two-way
379 ANOVA with Dunnett's multiple comparisons test (vs. IVIg/IdeS and PBS/IdeS); **i**, one-way
380 ANOVA with Tukey's multiple comparisons test.

381

382 **Figure 2** IdeS treatment enhances transduction in NHPs in the presence of pre-existing anti-
383 AAV NAbs. **a**, Experimental protocol. Two male Cynomolgus macaques with an anti-AAV8
384 NAb titer of 1:17.2 were infused with either saline (NHP3, control), or twice with 500 $\mu\text{g kg}^{-1}$
385 of IdeS (NHP5, treated) before the administration of an AAV8-hFIX vector. **b**, Total IgG over
386 time in NHP5 (n=1 animal, samples tested in duplicate). **c**, Western blot showing IgG
387 degradation over time in NHP5. One representative out of two independent experiments
388 shown. **d**, Effect of IdeS administration on anti-AAV8 IgG and **e**, NAbs (n=1 monkey per
389 treatment group, triplicate independent measurements of each sample). The horizontal dotted
390 lines represent the levels of anti-AAV8 IgG and NAbs in the control animal NHP3, see also
391 Supplementary Table 1. **f**, hFIX transgene levels in plasma over time (n=1 monkey per
392 treatment group, triplicate independent measurements of each sample). **g**, vector genome copy
393 number (VGCN) in liver at sacrifice (n=1 monkey per treatment group, average VGCN in
394 each of the four main liver sections). **h**, Anti-AAV8 IgG and **i**, anti-AAV8 NAbs at baseline
395 and post AAV8-hFIX vector administration (n=1 monkey per treatment group, duplicate
396 independent measurements of each sample). All data are shown as mean \pm s.d. Statistical
397 analyses were performed by **b,d,e**, repeated measures one-way ANOVA with Dunnett's
398 multiple comparisons test (vs. Day -2); **f,h,i**, repeated measures two-way ANOVA with
399 Sidak's multiple comparisons test (vs. NHP3); **g**, two-tailed unpaired t-test.

400

401 **Figure 3** IdeS treatment allows for AAV-LK03 vector readministration in NHPs. **a**,
402 Experimental protocol. Eight African green monkeys received 2×10^{12} vg kg⁻¹ of AAV-LK03-
403 GAA at Day 0. At Day 210, animals were given one dose of 1 mg kg⁻¹ IdeS (IdeS, n=5) or
404 vehicle control (PBS, n=3), followed by infusion of 2×10^{12} vg kg⁻¹ of AAV-LK03-hFVIII at
405 Day 211. **b,c**, Anti-AAV-LK03 IgG levels as a function of time in animals treated with
406 either PBS (**b**) or IdeS (**c**). **d,e**, Anti-LK03 NAb titers before (day 210) and after (day 211)
407 treatment with PBS (**d**) or IdeS (**e**). **f,g**, hFVIII antigen levels in plasma as a function of time
408 in animals treated either with PBS (**f**) or IdeS (**g**). **h**, Aggregate hFVIII expression was
409 quantitated using area under the curve (AUC) from day 211 to day 239 in PBS- and IdeS-
410 treated animals (bars and whisker indicate the mean and s.d.). Statistical analyses performed
411 by **d,e**, two-tailed ratio paired t-test. **h**, two-tailed Mann-Whitney U test.

412

413 **Figure 4** Efficient cleavage of human IgG in plasma in the presence of anti-IdeS antibodies.
414 Serum samples from 10 healthy donors and 12 subjects affected by Crigler-Najjar syndrome
415 were incubated *in vitro* with IdeS or PBS for 24 hours. **a**, Anti-AAV8 IgG and **b**, and anti-
416 IdeS IgG were then assessed by ELISA. **c**, Western blot analysis of cleavage of IgG by IdeS
417 in sera from 4 healthy donors (HD1-4) and 5 monkeys (NHP1, 2, 4, 5, 6) incubated with IdeS
418 (+) or PBS (-) for 24 hours. All data are shown as mean \pm s.d. Statistical analyses were
419 performed by two-tailed, paired Wilcoxon test.

420

421 **Methods**

422

423 **Care and use of animals**

424 Mouse studies were performed according to the French and European legislation on animal
425 care and experimentation (2010/63/EU and CE12-037) and approved by the local institutional
426 ethics committee (Protocols n 2017-014-B). All procedures using cynomolgus macaques
427 (*Macaca fascicularis*) were approved by the institutional animal care and use committee
428 (protocol number 2018120409565637, APAFIS 18092) and were conducted at Nantes
429 veterinary school (Oniris, Nantes, France). All procedures using African green monkeys
430 (*Chlorocebus sabaesus*) were conducted under IACUC approved protocols (# AC18175)
431 at the Saint Kitts Biomedical Research Foundation/Virscio (Saint Kitts and Nevis).

432

433 **Cloning and production of IdeS endopeptidase**

434 The IdeS coding sequence was cloned into a pEX-N-His-tagged cloning vector (OriGene
435 Technologies, Inc., Rockville MD), which was transformed into BL21 competent *E. coli*
436 (NEB, Ipswich, MA) following manufacturer's instructions. Protein expression was induced
437 at 37°C for 4 hrs by adding IPTG 0.5 mM to the bacterial culture. Detection and purification
438 of the protein were performed using the anti-FabRICATOR antibody A3-AF1-010 (Genovis,
439 Lund, Sweden) and a nickel affinity column (GE Healthcare, Chicago, IL), respectively.
440 Endotoxin removal was performed using poly(ϵ -lysine) resin spin columns (Thermo
441 Scientific, Waltham, MA) and residual endotoxin concentration was assessed by LAL test
442 with Endosafe Cartridge Technology (Charles River Laboratories, Wilmington, MA),
443 according to manufacturer's instructions. IdeS endopeptidase was diluted in the injectable
444 solution buffer, composed of 50 mM sodium phosphate, 150 mM NaCl, 0.25% human serum
445 albumin, pH 6.6.

446

447 **AAV vectors**

448 AAV vectors were prepared as previously described²⁶ in roller bottles following a triple
449 transfection protocol with subsequent cesium chloride gradient purification. The AAV vector
450 titration was performed by real time PCR (qPCR) performed in ABI PRISM 7900 HT
451 Sequence Detector using Absolute ROX mix (Taqman, Thermo Fisher Scientific, Waltham,
452 MA), except for empty capsids that were titrated by SDS-PAGE followed by silver staining
453 and quantification by densitometry against an AAV capsid used as a standard. For the *in vitro*
454 neutralization assay, an AAV8 or an AAV-LK03 vector encoding Firefly Luciferase (AAV8-
455 Luc or AAV-LK03-Luc) was used as reporter as previously described¹⁵. The AAV8 vectors
456 used in the *in vivo* experiments expressed human factor IX (hFIX) or Gaussia Luciferase
457 (GLuc), under the control of a liver-specific promoter^{4,17}. The AAV-LK03 vectors used for
458 the *in vivo* studies expressed the human alpha-acid glucosidase (AAV-LK03-GAA) or the
459 human factor VIII (AAV-LK03-hFVIII) transgene under the control of liver specific
460 promoters. These vectors were purified by a triple transfection as above followed by column
461 purification.

462

463 **Human serum samples**

464 Commercially available serum samples from deidentified healthy volunteers were obtained
465 from the Etablissement Français du Sang (EFS). Serum samples from patients with the
466 Crigler-Najjar (CN) syndrome were collected in the context of an observational international
467 multicenter study (NCT02302690) to study AAV seroprevalence in this patient population.
468 Anti-AAV antibodies in CN serum were assessed at Genethon (Evry, France). The study was
469 reviewed and approved by the independent ethics committees of the five participating sites
470 (Hôpital Antoine Bécclère, Clamart, France; Academic Medical Center, Amsterdam, the

471 Netherlands; Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy; Federico II
472 University Hospital of Naples, Naples, Italy; Hannover Medical School, Hannover, Germany)
473 and was carried out in compliance with the Good Clinical Practice guidelines and according
474 to the principles of the declaration of Helsinki. All participants gave written informed consent
475 prior to study enrollment.

476

477 **Cell culture**

478 The 2V6.11 cells (ATCC CRL-2784) were maintained under 37°C, 5% CO₂ condition in
479 Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM
480 GlutaMAX (Invitrogen Life Technology, Carlsbad, CA) and were used for the AAV
481 neutralization assay¹⁵.

482

483 **Capsid antibody ELISA**

484 Anti-AAV8 Ig capture assay was performed as previously described¹⁷. Briefly, recombinant
485 AAV8 empty capsids were diluted in coating buffer (35 mM bicarbonate, 13 mM carbonate,
486 pH > 9.2) to a final concentration of 2x10¹⁰ vector particles per ml. Fifty microliters were
487 added to each well in a 96-well Nunc Maxisorp Immunoplate (ThermoFisher, Waltham,
488 USA). A standard curve made from purified human IgG (IVIg, Tégéline, LFB, Les Ulis,
489 France) was added directly to the plates. Plates were coated overnight at 4°C. The next day,
490 plates were washed three times with wash buffer (PBS, 0.05% Tween-20) then blocked with
491 blocking buffer (PBS, 6% fat-free milk) for 2 hrs at room temperature. Plates were again
492 washed three times with wash buffer then incubated with serum diluted in blocking buffer for
493 1 hr at 37°C. After 3 washes, horseradish peroxidase (HRP)-conjugated antibodies specific
494 for the heavy chain of human IgG (Southern Biotech, Birmingham, US-AL) were incubated
495 for 1 hr at 37°C. Plates were then washed three times with wash buffer and revealed with o-

496 phenylenediaminedihydrochloride (OPD) substrate (Sigma, Saint Louis, USA). Optical
497 density (OD) was measured at 492 nm using a microplate reader (ENSPIRE™, Perkin Elmer,
498 Waltham, USA). Anti-AAV8 IgG concentration was determined against the specific standard
499 curve using 4-parameters regression and results were expressed as µg/ml of IgG.
500 For NHP samples, a purified monkey IgG or IgM for the standard curves and an HRP-
501 conjugated antibody specific for gamma chain of monkey IgG or IgM (Fitzgerald Industries,
502 Acton, MA) for the revelation, were used.
503 The anti-AAV-LK03 IgG and IgM capture assay was performed as described above with the
504 following modifications in the protocol. Plates were coated with AAV-LK03 empty capsid at
505 1.57×10^{11} capsid particles per ml (50 µl total) in coating buffer. Detection of bound IgG was
506 performed with an HRP-conjugated goat anti-monkey IgG, Fc-specific polyclonal antibody
507 (GAMon/Ig(Fc)/PO, Nordic MUBio, Susteren, The Netherlands) at a 1:5,000 dilution.
508 Detection of bound IgM was performed with an HRP-conjugated polyclonal goat anti-
509 monkey IgM (43R-IG074HRP, Fitzgerald Industries International, Acton, MA) at a 1:10,000
510 dilution. For IgG, 7-point standard curve was generated by passively absorbing purified
511 monkey IgG (Rockland Immunochemicals, Limerick, PA) onto duplicate wells in place of the
512 AAV coating step at concentrations ranging from 2 µg/ml to 31.25 ng/ml. For IgM, purified
513 monkey IgM (Fitzgerald Industries International) were used at concentrations ranging from
514 250 to 1.95 ng/ml.

515

516

517 ***In vitro* AAV8 neutralization assay**

518 The NAb assay was performed as previously described¹⁵. Briefly, on day 1, 96-well plates
519 were seeded with 2×10^4 2V6.11 cells/well for 24 hrs in the presence of ponasterone A (Life
520 Technologies, Carlsbad, USA). An AAV8-Luc or an AAV-LK03-GLuc vector was then

521 diluted in serum-free DMEM (Life Technologies, Carlsbad, USA) and incubated with semi-
522 log-fold serial dilutions of the serum samples for 1 hr at 37°C. Subsequently, the serum-
523 vector mixtures were added to the cells at an multiplicity of infection (MOI) of 200. After
524 24 hrs, cells were lysed and the luciferase activity was measured on a luminometer
525 (ENSPIRE™, Perkin Elmer, Waltham, MA). For the AAV-LK03-GLuc vector, cells were not
526 lysed and luciferase activity measured on cell supernatant. Luciferase expression was
527 measured as Relative Light Unit (RLU) per second. The neutralizing titer was reported as the
528 highest serum dilution that inhibited AAV transduction by $\geq 50\%$ compared to the 100%
529 transduction control.

530

531 ***In vitro* incubation of IgG and AAV vector with IdeS**

532 Human sera from AAV8-seropositive healthy donors or CN subjects²⁵ were diluted at 1:3 in
533 PBS and incubated with or without IdeS (200 IU, 4 μg) at 37°C for 24 hrs. Intact anti-AAV8
534 IgG and anti-IdeS IgG were assessed by ELISA or by Western Blot as described.

535 For the *in vitro* incubation of AAV8 vectors with IdeS, 1.5×10^{11} vg of AAV8-CMV-Luc were
536 mixed with increasing amounts of IdeS (0, 0.6, 3, or 6 μg) in a total volume of 50 μl and
537 incubated at 37°C during for 24h. An untreated AAV8-CMV-Luc vector without incubation
538 at 37°C was used as control to check the effect of a prolonged incubation at 37°C on
539 transduction efficiency. After incubation, vectors were added to HEK 293 cells (seeded in 96
540 well plates at a concentration of 2×10^4 cells/well) at an MOI of 100,000, 10,000, or 1,000.
541 After 30 hours, cells were lysed and the luciferase activity was measured on a luminometer.
542 Luciferase expression was measured as Relative Light Unit (RLU) per second.

543

544 **Mouse studies**

545 Wild-type male C57BL/6 mice or factor IX (FIX)-deficient hemophilia B (HB) male and
546 female mice, all aged 6-8 weeks, were used in these studies. In all experiments, animals were
547 passively immunized by intravenous injection of 9 mg of IVIg (Privigen, CSL-Behring, King-
548 of-Prussia, PA USA) in a final volume of 100 μ l. After 30 min, mice received 25 μ g of IdeS
549 by retro-orbital injection. Twenty-four hours later, mice were injected with an AAV8 vector
550 at the dose of 1×10^{12} vg/kg (2×10^{10} vg/mouse) by tail vein route. Blood samples were
551 collected via mandibular puncture or from the retro-orbital plexus in heparin coated capillary
552 tubes (Sarstedt, Nümbrecht, Germany). At euthanasia, whole livers were collected and snap-
553 frozen for subsequent assessment of vector genome copy numbers (VGCN).

554

555 **Tail clip bleeding assay**

556 HB mice were anesthetized with a mixture of ketamine and xylazine delivered
557 intraperitoneally and placed in prone position. A distal 3 mm segment of the tail was
558 amputated with a scalpel. The tail was immediately immersed in a 50 ml tube containing
559 isotonic saline pre-warmed at 37°C. The position of the tail was vertical with the tip
560 positioned about 2 cm below the body horizon. Each animal was monitored for 20 minutes
561 even if bleeding ceased, in order to detect any re-bleeding. Bleeding time was determined
562 using a stop clock. If bleeding on/off cycles occurred, the sum of bleeding times within the
563 20-minute period was used. At the end of the experiment, animals were sacrificed for liver
564 VGCN analysis.

565

566 **NHP studies**

567 Cynomolgus monkeys:

568 Six Cynomolgus monkeys (*Macaca fascicularis*, 2-3 years old), were selected based on their
569 anti-AAV8 neutralizing antibody titer (between 1:3.16 to 1:31.6). The study plan was divided

570 into 3 sub-studies and monkeys were distributed as treated and untreated groups, each group
571 having similar anti-AAV8 NAb titers.

572 The injection protocol was performed as follows. IdeS was administered via slow intravenous
573 infusion at the dose of 500 µg/kg. Thirty minutes before IdeS injection, an antihistamine drug
574 (Polaramine, 4mg/kg) was administered. The AAV8-hFIX vector was infused 24 hrs after the
575 last IdeS injection. Blood samples were collected before and immediately after IdeS
576 treatment, and before AAV administration to assess the efficiency of degradation of IgG.
577 Other blood samples were collected throughout the protocol in order to follow up the anti-
578 AAV8 humoral response, transgene expression as well as to monitor hematology and clinical
579 chemistry parameters. At sacrifice, 4 wedge biopsies were taken at different sites from the 4
580 liver lobes, right, left, caudate and papillary process, to assess VGCN in different areas of the
581 liver. Animals were also submitted to full necropsy including a macroscopic examination and
582 liver histopathological evaluation.

583

584 African green monkeys:

585 Eight adult male African green monkeys (*Chlorocebus sabaeus*), confirmed seronegative for
586 AAV-LK03 neutralizing antibodies, were enrolled in the study. At day 0, animals were
587 immunized with an AAV-LK03 vector encoding for alpha-acid glucosidase (AAV-LK03-
588 GAA)²⁷. At study day 210, 5 animals were randomly assigned to receive a single infusion of
589 IdeS delivered via infusion pump a dose of 1 mg/kg, while 3 animals received vehicle control
590 (saline). Approximately 15-30 minutes prior to infusion of IdeS or PBS animals were treated
591 with diphenhydramine (5 mg/kg, injected intramuscularly). 24 hours after dosing with IdeS or
592 vehicle, animals were infused with an AAV-LK03 vector encoding for human factor VIII
593 (AAV-LK03-FVIII), at a dose of 2×10^{12} vg/kg, through the saphenous vein. Plasma samples
594 were collected at day 0, 1 hour, 2 hours, 6 hours, 12 hours, 24 hours, 48 hours, and 8, 15, 22,

595 and 29 days after IdeS infusion. Animals were euthanized following the Day 29 plasma
596 collection.

597

598 **IdeS clearance**

599 A kinetic study was conducted in 2 Cynomolgus monkeys following a protocol similar to
600 what previously described in humans¹⁹. Animals received 500 µg/kg of IdeS intravenously at
601 time 0 and 24 hours later and IdeS level in blood was measured by sandwich ELISA over
602 time. Briefly, plates were coated with a polyclonal anti-FabRICATOR antibody (A3-AF1-
603 010, Genovis, Lund, Sweden) and blocked with PBS, 3% BSA. Plasma samples were then
604 added to the plates and incubated for 1 hour at room temperature. Purified IdeS was used as a
605 standard. A biotinylated anti-HIS tag antibody (AD1.1.10, R&D Systems, Minneapolis, MN)
606 and streptavidin-conjugated HRP (Dako, Santa Clara, CA) were used for detection. TMB
607 substrate (BD Biosciences, Franklin Lakes, NJ) were used for quantification and the reaction
608 was stopped with a 3M H₂SO₄ solution and optical density (OD) measurements were done at
609 450 nm. IdeS concentration was determined against the specific standard curve using 4-
610 parameters regression and results were expressed as µg/ml.

611

612 **Anti-monkey IgG ELISA**

613 Plates were coated with a goat anti-human IgG F(ab')₂ (Thermo Fisher, Waltham, MA, US)
614 and blocked with PBS-6% milk. Diluted samples were incubated and a commercial purified
615 monkey IgG (Fitzgerald Industries, Acton, MA) was used as a standard. Intact monkey IgG
616 were then revealed using a secondary goat anti-monkey HRP-conjugated IgG (Fitzgerald
617 Industries, Acton, MA) and the OPD substrate. The reaction was stopped with 3 M H₂SO₄ and
618 OD was measured at 492 nm.

619

620 **Human FIX ELISA**

621 Human FIX (hFIX) protein levels in mouse plasma were measured by ELISA using a capture
622 antibody and a HRP-conjugated detection antibody (Affinity Biological, Ancatser, Canada),
623 following the manufacturer's instructions. In NHP samples, anti-hFIX antibody (MAI-43012,
624 Thermo Fisher Scientific Waltham, MA), and an anti-hFIX-HRP antibody (CL20040APHP,
625 Tebu-bio, Le Perray-en-Yvelines, France) were used for capture and detection, respectively.

626

627 **Human Factor VIII ELISA**

628 The levels of transgene-derived human FVIII in plasma were quantified using a sandwich
629 ELISA. Briefly, 96-well microplates (Sigma Aldrich, Saint Louis, MO) were coated with
630 FVIII capture antibody (GMA-8024, Green Mountain Antibodies, Burlington, VA) at 2 µg/ml
631 (100 µL total) in coating buffer and incubated overnight at 4°C. After coating, plates were
632 washed, blocked for 1 hour at room temperature, and samples were added to each well in
633 duplicate. An 8-point standard curve was created by spiking in recombinant B-domain deleted
634 human FVIII (Xyntha, Pfizer, New York, NY) into FVIII-depleted human plasma (George
635 King BioMedical, Overland Park, KS) at known concentrations ranging from 300 to 1.09
636 ng/ml in duplicate wells. Standards and diluted samples were added to wells at a volume of
637 100 µl and incubated for 1 hour at 37°C. Following sample incubation, plates were washed,
638 and incubated with a biotinylated mouse anti-human Factor VIII antibody (GMA-8023, Green
639 Mountain Antibodies) at a dilution of 1:5000 (100 µl). Plates were washed and chromogenic
640 signal was generated by adding 100 uL of TMB substrate to each well, followed by quenching
641 with 100 µl 1N sulfuric acid. Absorbance was measured using an i3 SpectraMax (Molecular
642 Devices, San Jose, CA) at 450nm and FVIII antigen levels were extrapolated from the
643 standard curve. The lower limit of quantitation for this assay was 4.6 ng/ml.

644

645 **Vector genome copy number determination**

646 Liver VGCN was determined using a qPCR assay with primers and probes specific for the
647 liver promoter and the mouse titin or globin genes. The promoter-specific primers and the
648 probe²⁷ were purchased from Thermo Scientific (Waltham, MA, USA). Mouse titin and
649 globin were used as a normalizing gene in mice and NHP studies, respectively, and were
650 purchased from Thermo Scientific. DNA from tissues was extracted from whole-organ
651 (mouse) or wedge biopsies followed by homogenization using FastPrep (MP Biomedicals, ,
652 Irvine, CA) and Gentra Puregen Blood Kit (Qiagen, Hilden, Germany) DNA extraction. Each
653 sample was tested in triplicates and genome copy number per diploid genome was determined
654 against a standard curve made of linearized plasmid.

655

656 **Gaussia Luciferase assay**

657 Fifty microliters of plasma samples diluted in PBS (1:50) were loaded on white 96-well
658 microplate (OptiPlate-96 Perkin Elmer, Waltham, USA). Gaussia Luciferase activity was
659 measured on a Luminometer (ENSPiRE™, Perkin Elmer, Waltham, USA). The measurement
660 was done over 1 second after automatic injection of 50 µl of substrate solution
661 (Coelenterazine, Sigma Aldrich, Saint Louis, USA). Signal background was subtracted from
662 each value. The GLuc activity is expressed as RLU/sec and the values are the mean ± s.d. of
663 duplicates from repeated assays.

664

665 **Western blot for human and NHP IgG**

666 Western blots were performed on diluted samples, (1:20) for serum from passively
667 immunized mice or from NHPs. SDS-PAGE was performed in 4-12% gradient Bis-Tris
668 protein gels (Invitrogen). After protein transfer with semi-dry iBlot system (Invitrogen),
669 membranes were blocked with Odyssey buffer (Li-Cor Biosciences) overnight at 4°C. The

670 membranes were incubated with IgG F(ab')₂-specific antibody (Thermo Scientific, Waltham,
671 US-MA) or IgG Fc-specific antibody (Sigma, Saint Louis, USA), or with the anti-
672 FabRICATOR anti-IdeS antibody. The membranes were washed and incubated with the
673 appropriate secondary antibody (Li-Cor Biosciences) and visualized by Odyssey Imaging
674 System and the Image Studio Lite (v5.2) software (Li-Cor Biosciences).

675

676 **Anti-IdeS IgG ELISA**

677 Plates were coated overnight at 4°C with 3 µg/ml of IdeS. The next day, plates were blocked
678 and diluted samples were incubated for 1 hr at 37°C. A secondary HRP-conjugated antibody
679 specific for human (Southern Biotech, Birmingham, AL) or monkey IgG (Fitzgerald
680 Industries, Acton, MA) was incubated for 1 hr at 37°C and revealed with OPD substrate. The
681 standard curve in the assay was made of IVIg or purified monkey IgG (Fitzgerald Industries,
682 Acton, MA) serially diluted from a concentration of 2000 ng/ml. Anti-IdeS IgG concentration
683 was determined a using 4-parameters regression and results are expressed as µg/ml of IgG.

684

685 **Anti-hFIX and anti-hFVIII IgG ELISA**

686 Anti-hFIX IgG were determined as described²⁸. Plates were coated overnight at 4°C with 5
687 µg/ml of recombinant hFIX (Benefix, Pfizer, Paris, France). A standard curve made of serial
688 dilution of purified monkey IgG (Fitzgerald Industries, Acton, MA) was used as a reference to
689 extrapolate the concentration of anti-hFIX antibodies. The next day, plates were blocked and
690 diluted NHP sera were incubated for 1 hr at 37°C. A secondary HRP-conjugated antibody
691 specific for monkey IgG (Fitzgerald Industries, Acton, MA) was incubated 1 hr at 37°C and
692 revealed with OPD substrate. Anti-hFIX IgG concentration was determined using a 4-
693 parameters regression and results were expressed as µg/ml of IgG.

694 The presence and amount of anti-transgene antibodies developed against hFVIII was
695 quantified using an indirect ELISA. Briefly, 96-well microplates were coated with Xyntha
696 (Pfizer) at 1 µg/ml (100 µl total) in coating buffer overnight at 4°C. Following coating, plates
697 were washed, blocked for 30 hours at room temperature, and then incubated with plasma
698 samples for 1 hour at 37°C. Sample wells were prepared in duplicate. After sample
699 incubation, plates were washed and incubated in an HRP-conjugated polyclonal sheep anti-
700 human IgG (NA933, VWR, Radnor, PA) at a 1:5,000 dilution for 1.5 hours at RT.
701 Chromogenic signal was generated by adding 100 µl of TMB substrate to each well and
702 incubating at room temperature for approximately 10 minutes, followed by quenching with
703 100 µl 1N sulfuric acid. An 8-point standard curve was generated by diluting human plasma
704 with FVIII inhibitors (George King BioMedical) in naïve monkey plasma onto duplicate wells
705 to achieve inhibitor levels ranging from 16 to 0.125 arbitrary units. Absorbance was measured
706 on an i3 SpectraMax (Molecular Devices) at 450nm absorbance and anti-hFVIII IgG levels
707 were extrapolated from the standard curve and anti-hFVIII IgG levels are reported in arbitrary
708 units.

709

710 **Statistical analyses**

711 Data are represented as group mean ± s.d. Statistical significance ($\alpha = 0.05$) was tested using
712 GraphPad Prism (v7.0) and using different statistical methods depending on the dataset to be
713 analyzed and the experimental setup, statistical methods applied are specified in the figure
714 legends. P values below 0.05 were considered significant. When applicable, two-sided
715 statistical tests were used. For experiments involving a single NHP per treatment arm,
716 samples were measured multiple times in separate independent experiments and resulting data
717 used for statistical analysis. For mouse studies, animals were assigned randomly to treatment

718 groups. For NHP studies, animals were pre-selected based on the anti-AAV neutralizing
719 antibody titers and randomly assigned to the IdeS or control treatment arm of the experiment.
720

721 **Data availability statements**

722

723 All requests for raw and analyzed data and materials will be promptly reviewed by Genethon,
724 INSERM, or Spark Therapeutics to verify if the request is subject to any intellectual property
725 or confidentiality obligations. Patient-related data not included in the manuscript were
726 generated as part of a clinical trial and are not available as subjected to patient confidentiality.
727 Any data and materials that can be shared will be released via a Material Transfer Agreement
728 established with the relevant institution that generated the data or owns the materials.

729

730

731 **References Methods section**

732

733 4 Manno, C. S. *et al.* Successful transduction of liver in hemophilia by AAV-Factor IX
734 and limitations imposed by the host immune response. *Nat Med* **12**, 342-347,
735 doi:nm1358 [pii] 10.1038/nm1358 (2006).

736 15 Meliani, A. *et al.* Determination of anti-adenovirus vector neutralizing
737 antibody titer with an in vitro reporter system. *Hum Gene Ther Methods*,
738 doi:10.1089/hgtb.2015.037 (2015).

739 17 Mingozzi, F. *et al.* Overcoming preexisting humoral immunity to AAV using capsid
740 decoys. *Sci Transl Med* **5**, 194ra192, doi:10.1126/scitranslmed.3005795 (2013).

741 19 Lorant, T. *et al.* Safety, immunogenicity, pharmacokinetics, and efficacy of
742 degradation of anti-HLA antibodies by IdeS (imlifidase) in chronic kidney disease
743 patients. *Am J Transplant* **18**, 2752-2762, doi:10.1111/ajt.14733 (2018).

744 25 Aronson, S. J. *et al.* Prevalence and Relevance of Pre-Existing Anti-Adeno-Associated
745 Virus Immunity in the Context of Gene Therapy for Crigler-Najjar Syndrome. *Hum*
746 *Gene Ther* **30**, 1297-1305, doi:10.1089/hum.2019.143 (2019).

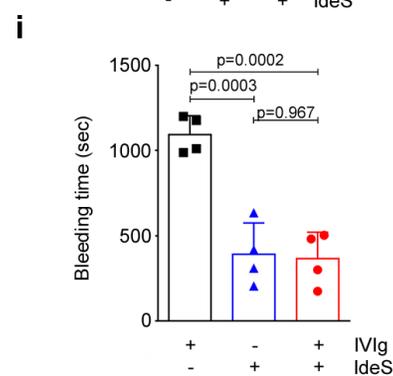
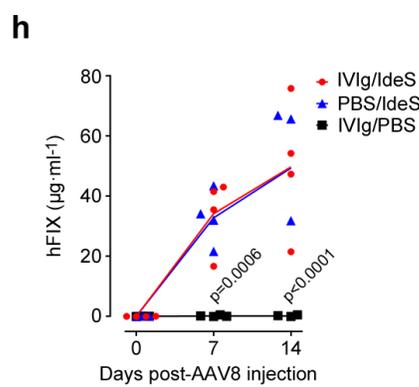
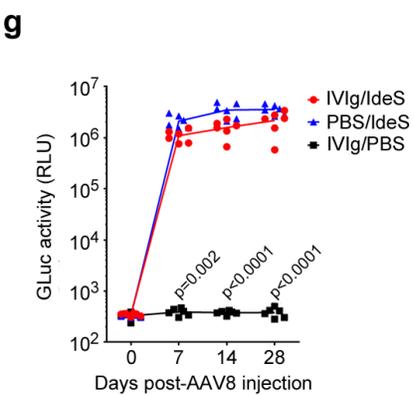
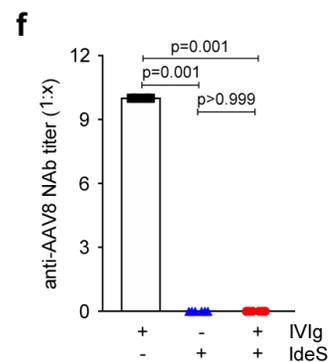
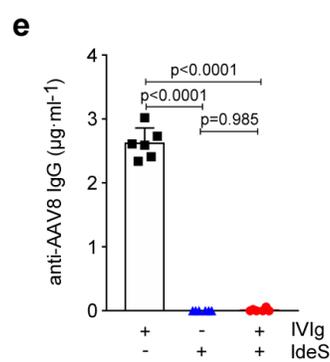
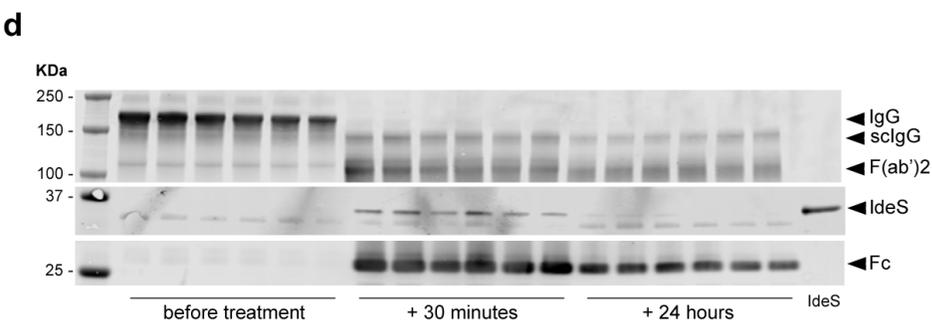
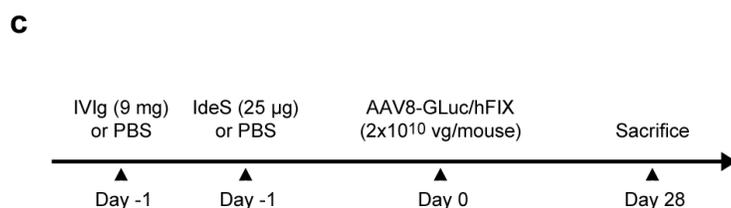
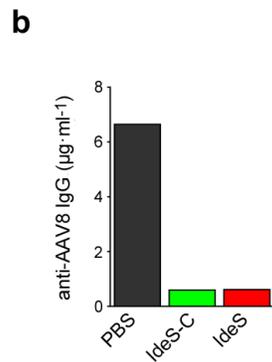
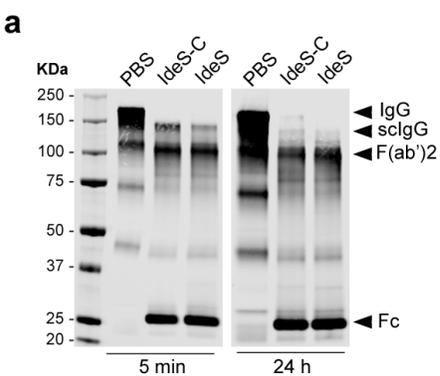
747 27 Puzzo, F. *et al.* Rescue of Pompe disease in mice by AAV-mediated liver delivery of
748 secretable acid alpha-glucosidase. *Sci Transl Med* **9**,
749 doi:10.1126/scitranslmed.aam6375 (2017).

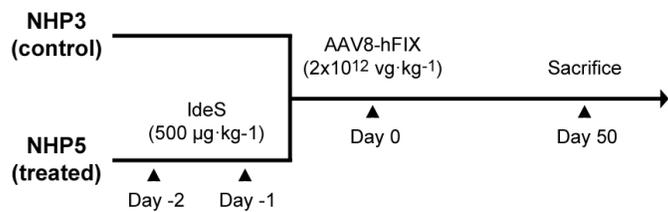
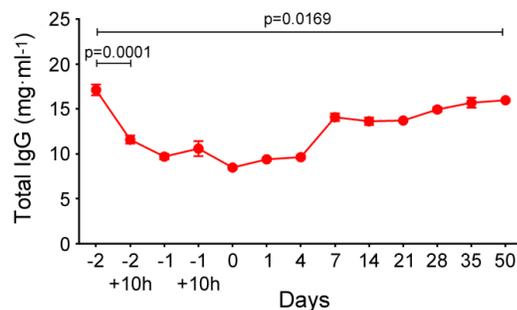
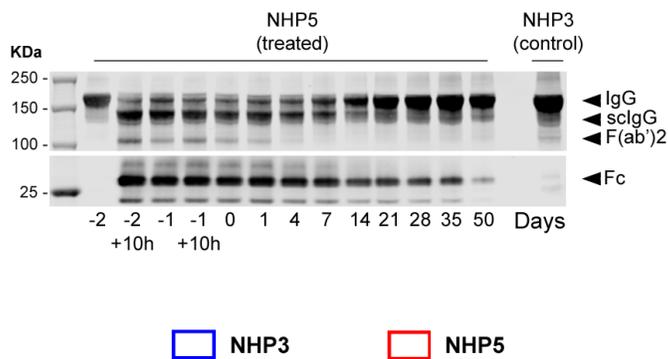
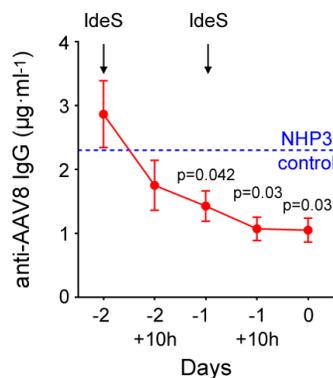
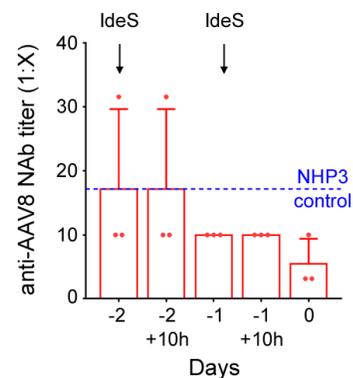
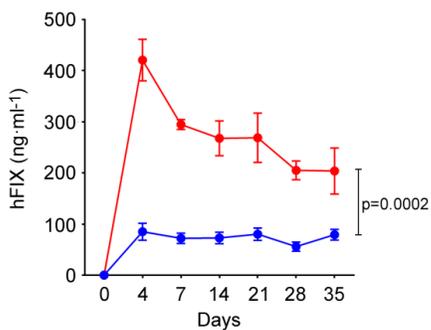
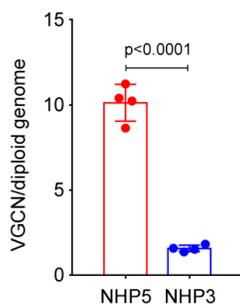
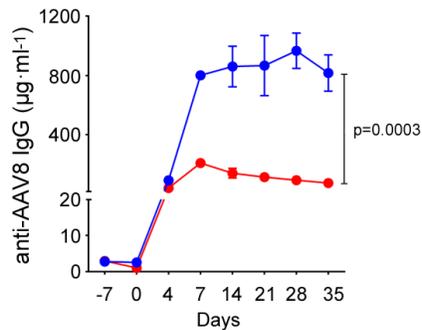
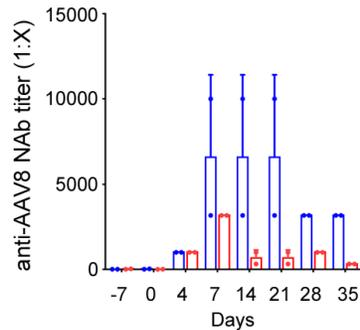
750 28 Mingozzi, F. *et al.* Modulation of tolerance to the transgene product in a nonhuman
751 primate model of AAV-mediated gene transfer to liver. *Blood* **110**, 2334-2341,
752 doi:blood-2007-03-080093 [pii] 10.1182/blood-2007-03-080093 (2007).

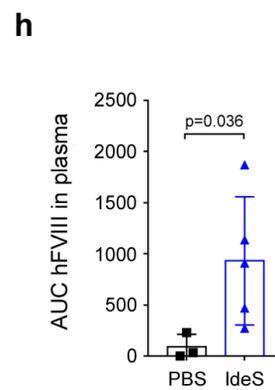
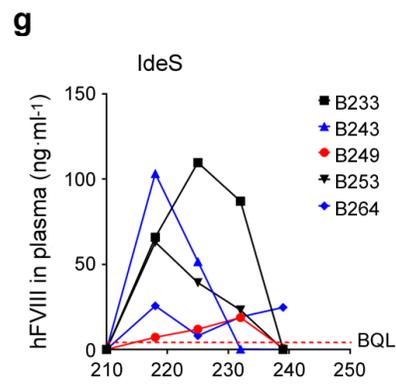
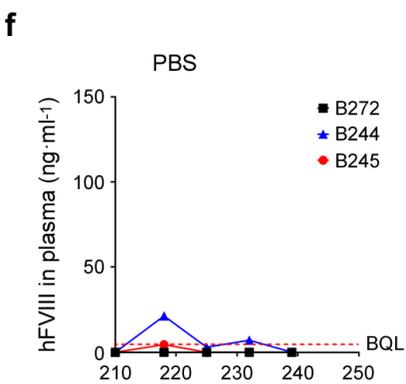
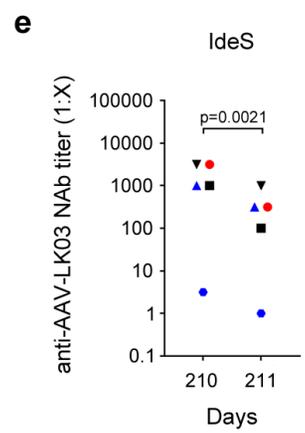
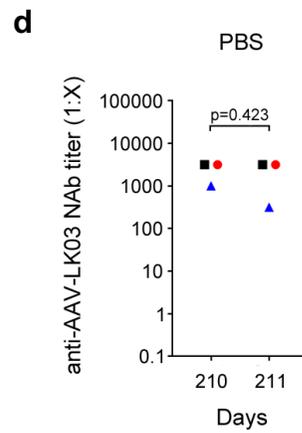
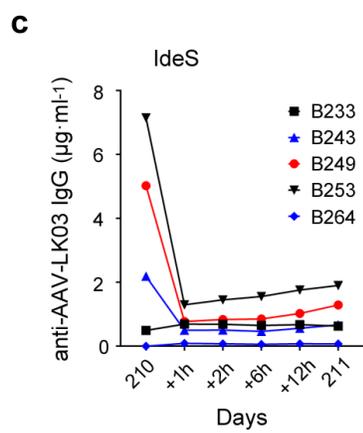
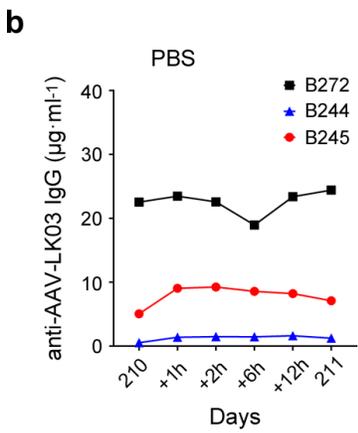
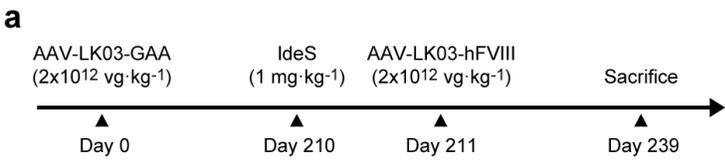
753

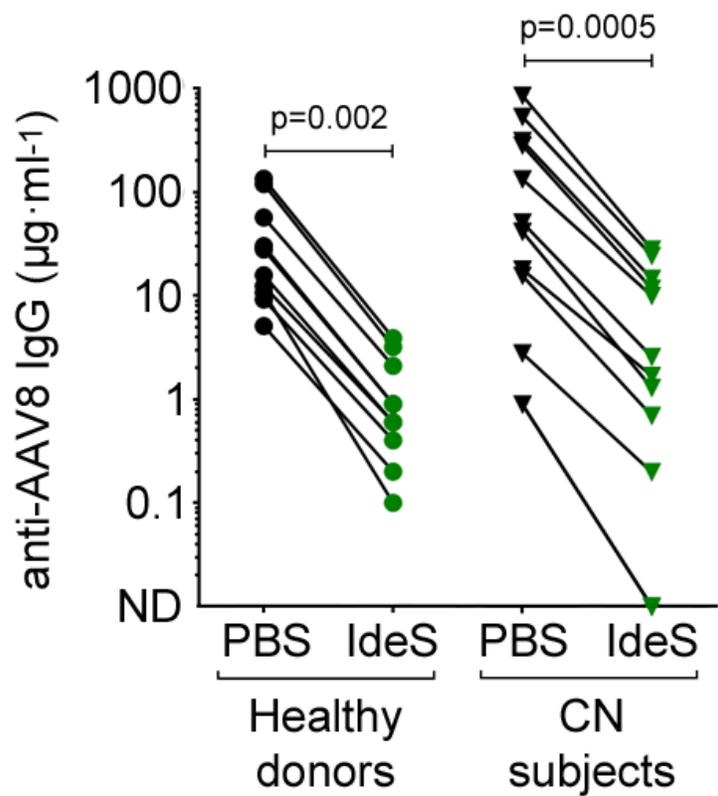
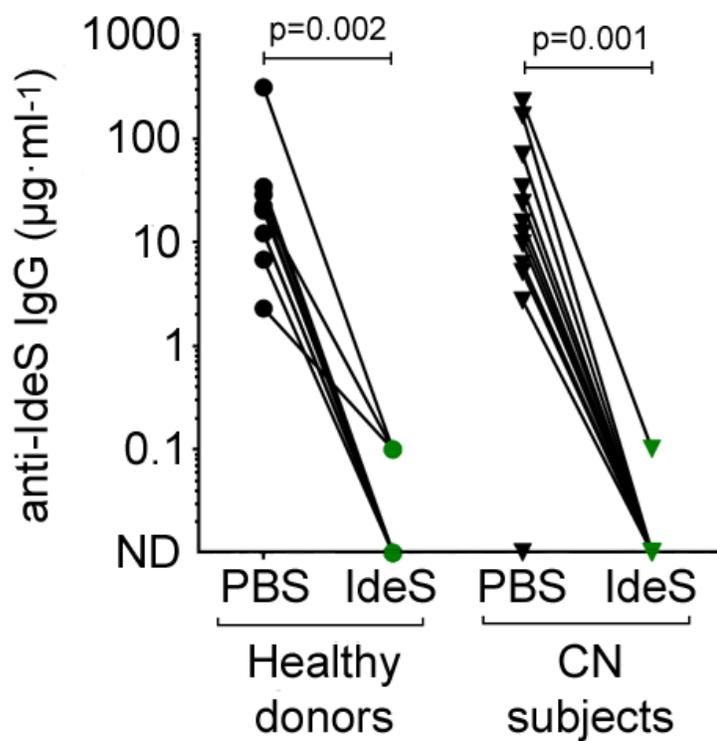
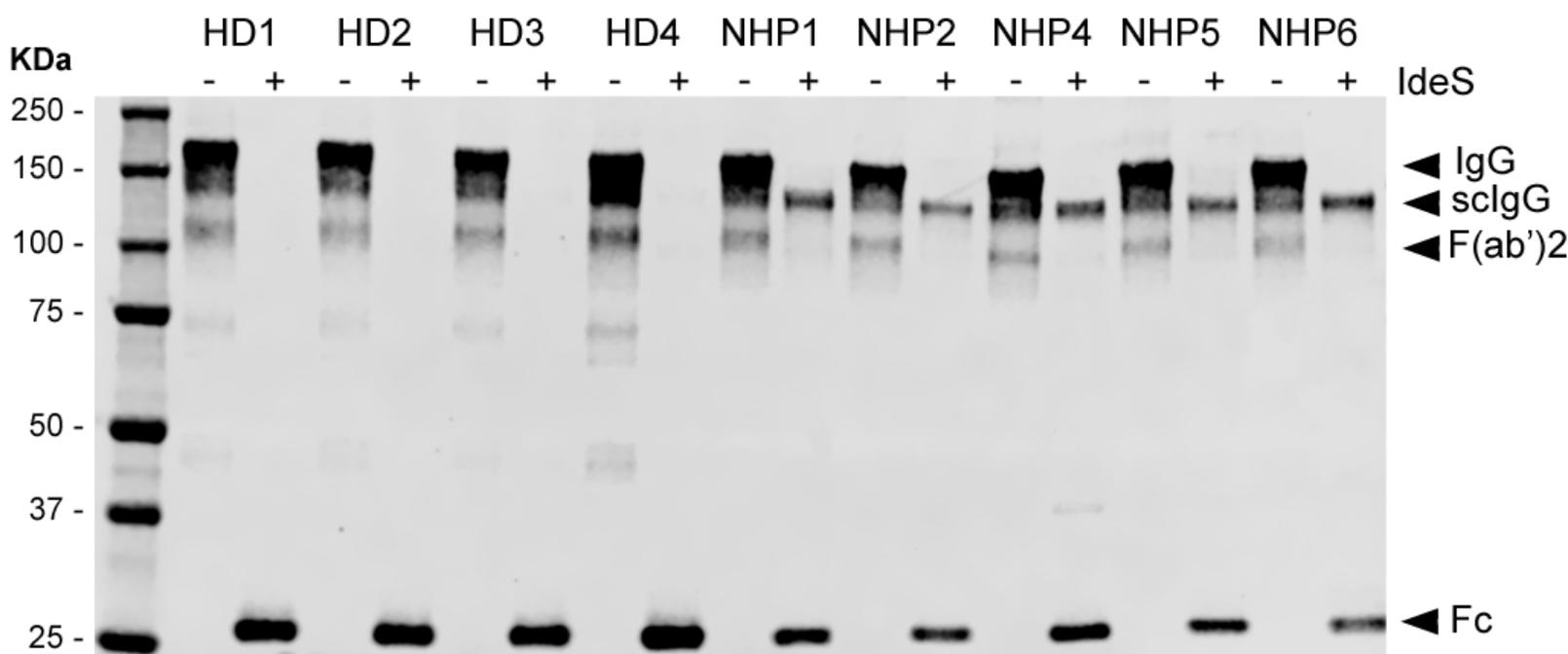
754

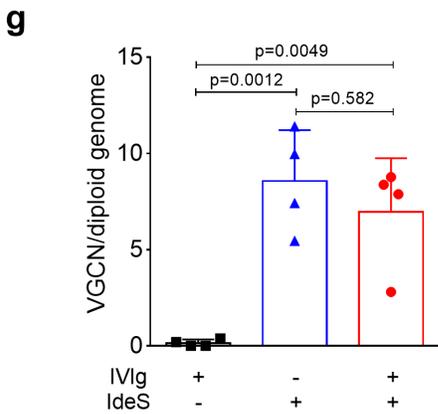
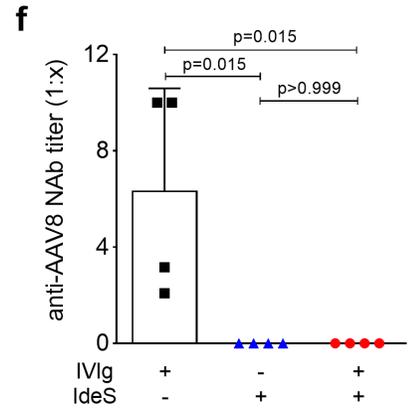
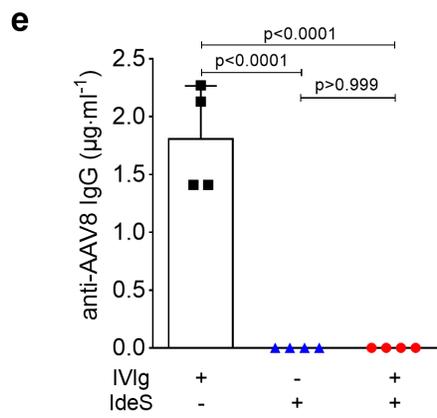
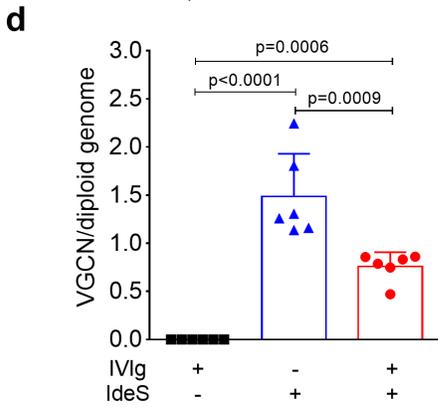
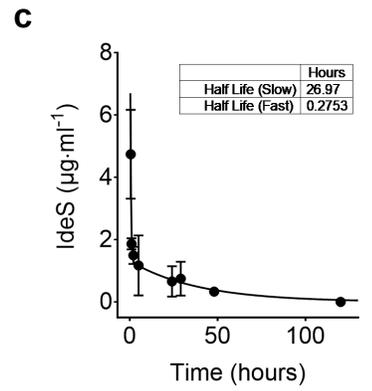
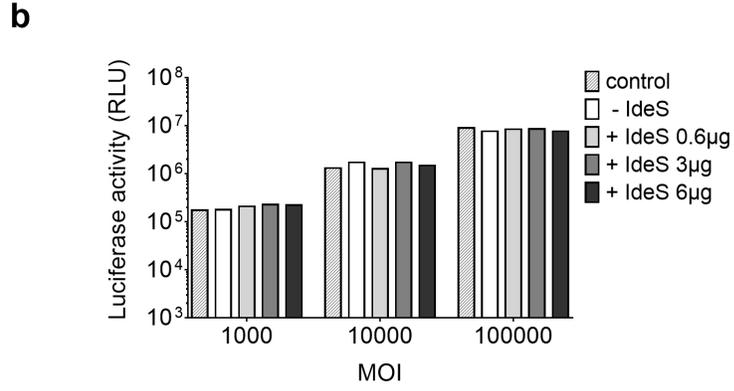
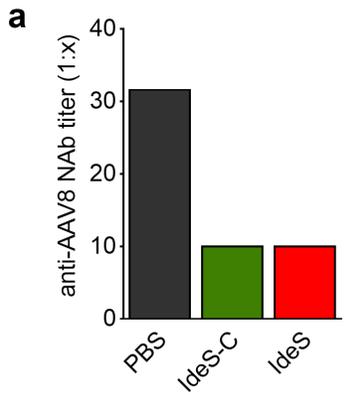
755

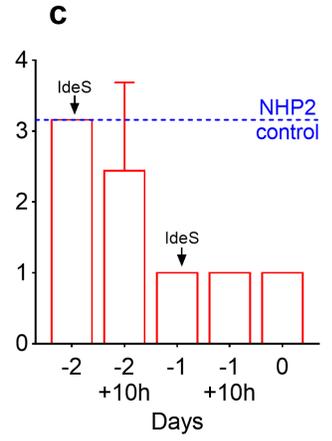
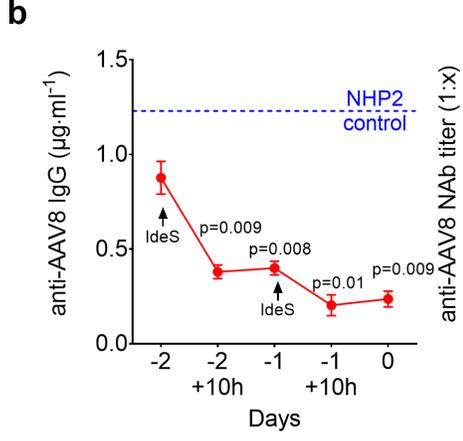
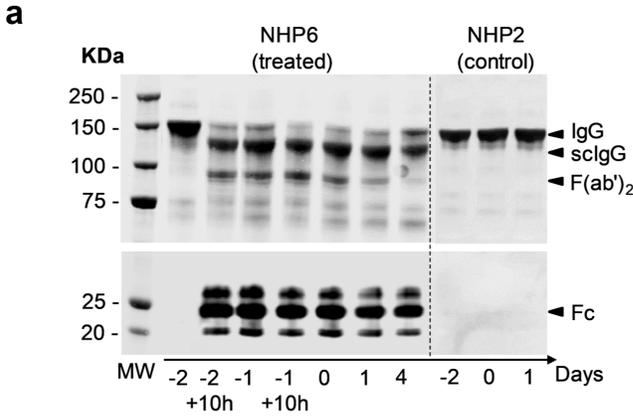


a**b****c****d****e****f****g****h****i**

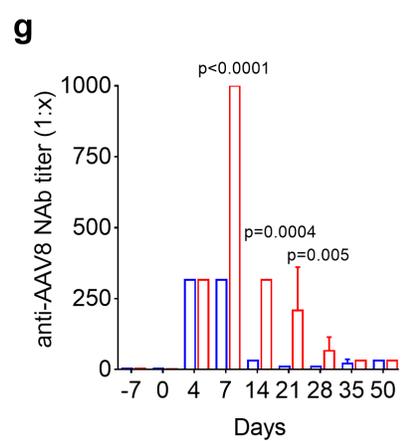
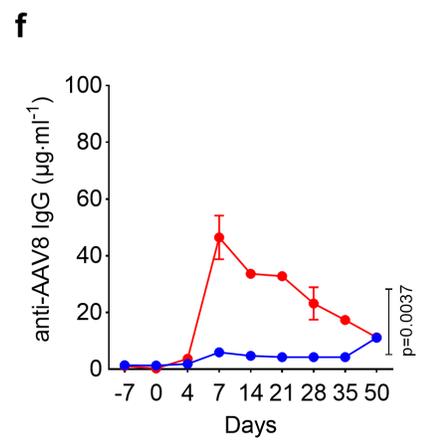
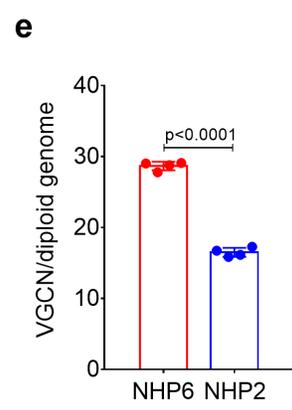
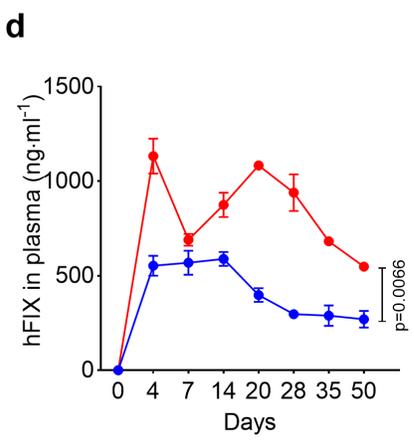


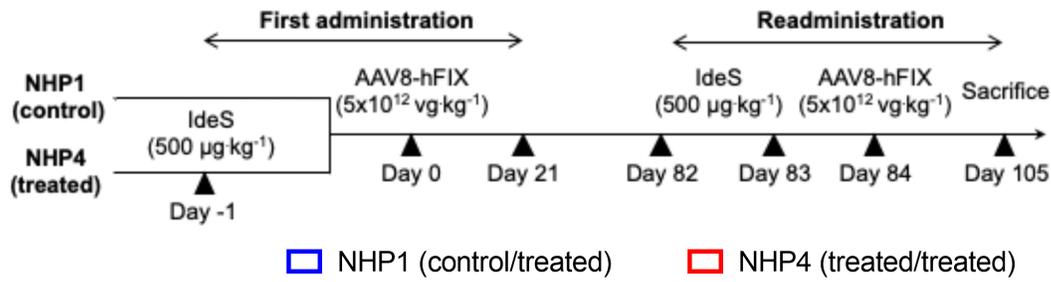
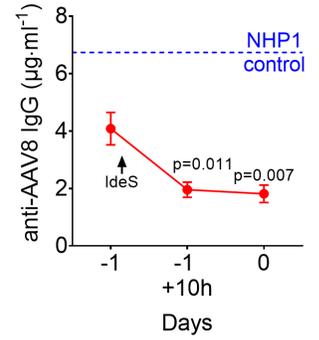
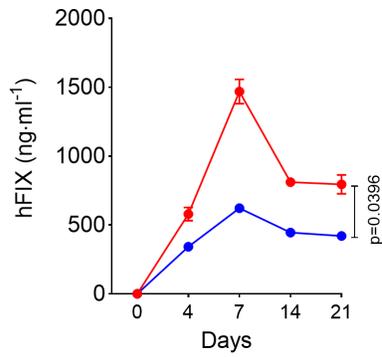
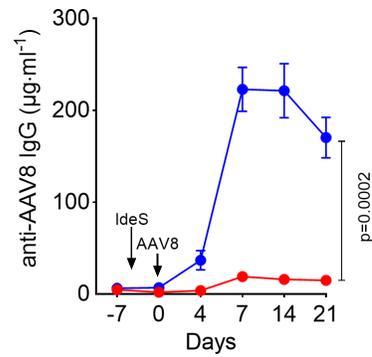
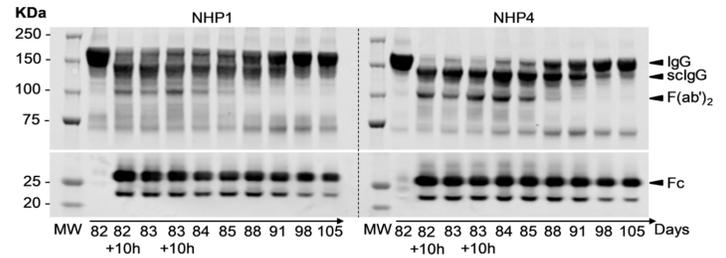
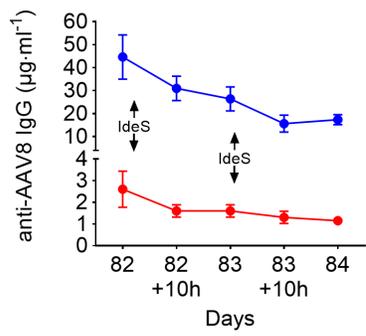
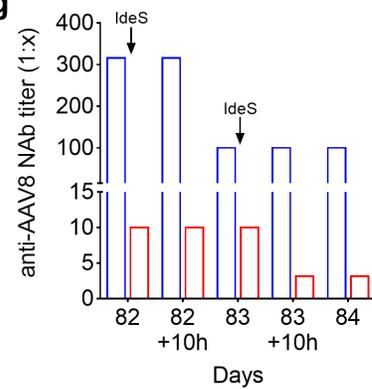
a**b****c**

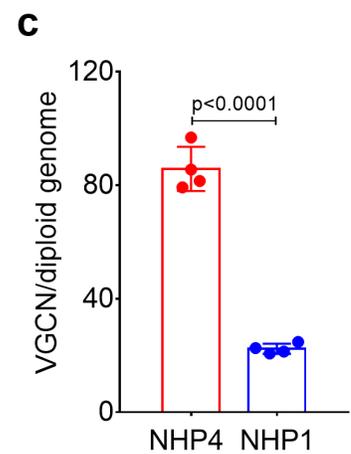
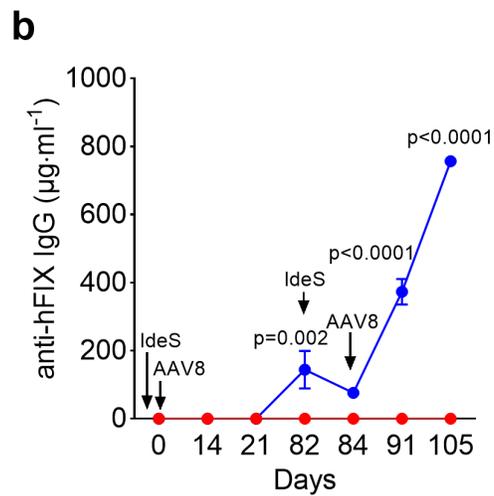
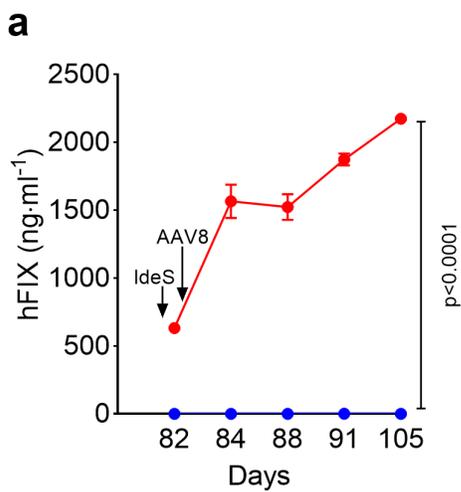




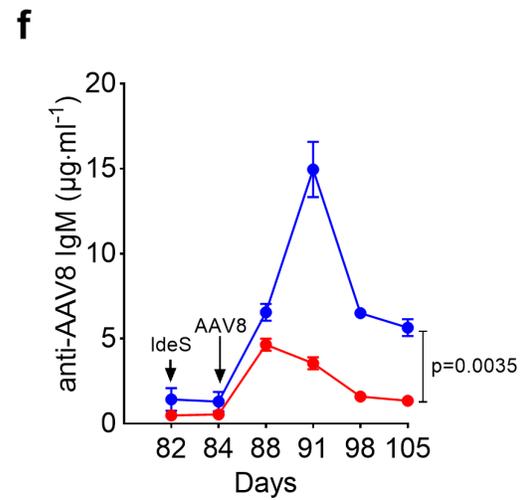
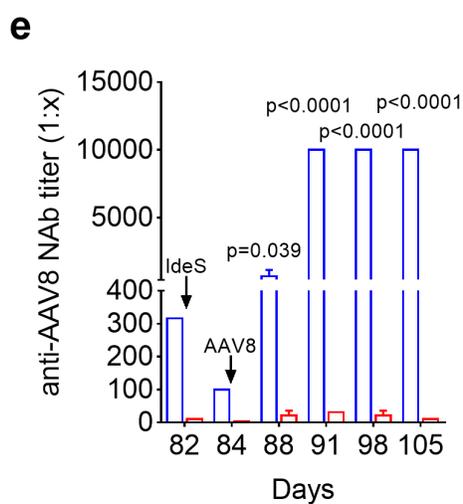
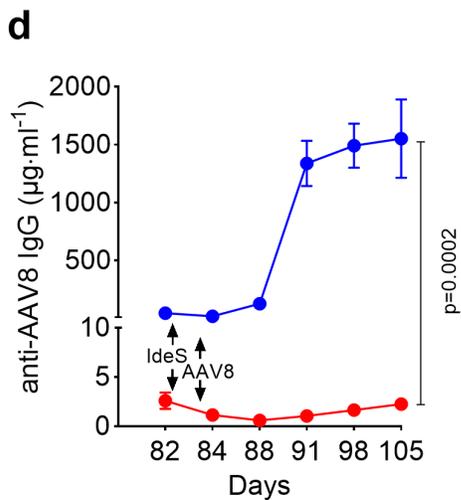
□ NHP2 (control) □ NHP6 (treated)

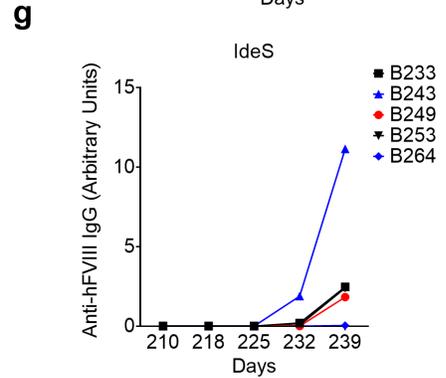
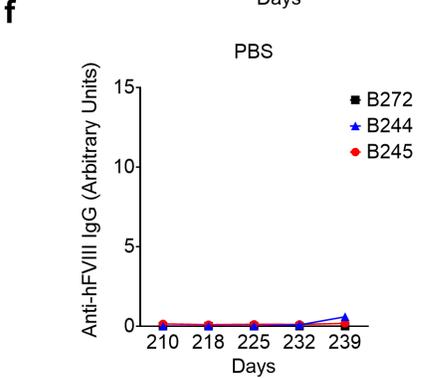
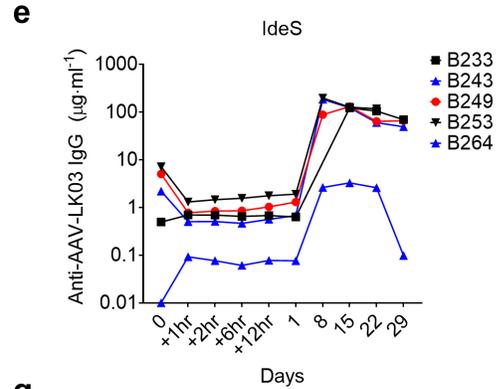
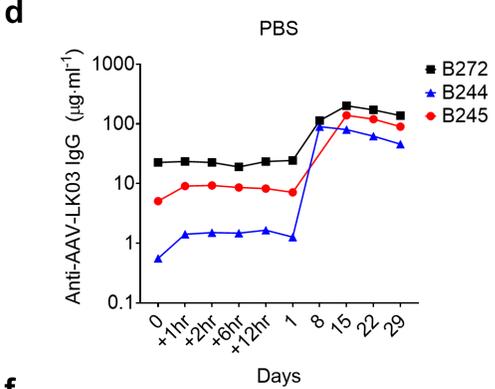
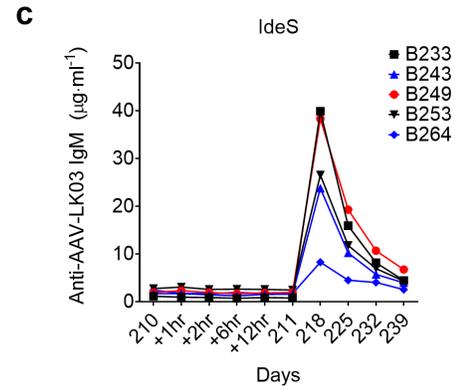
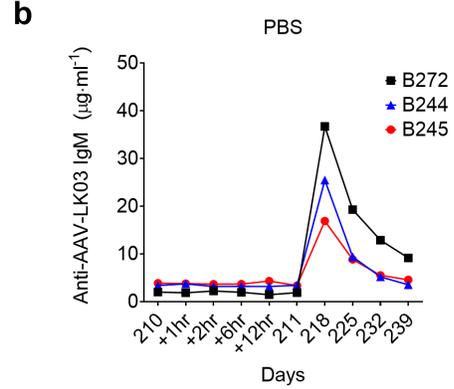
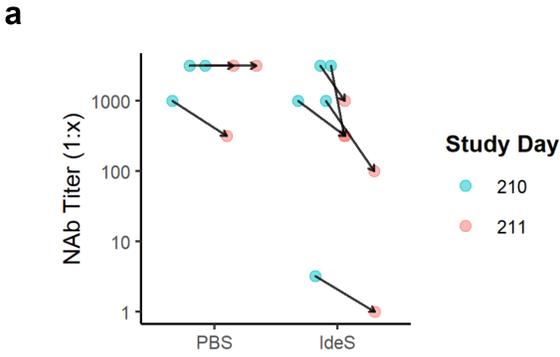


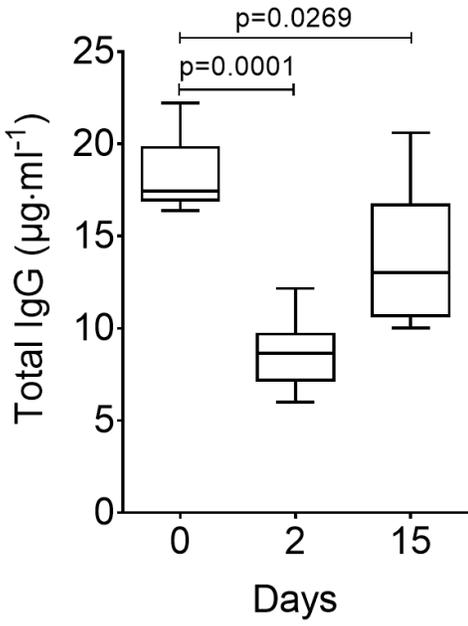
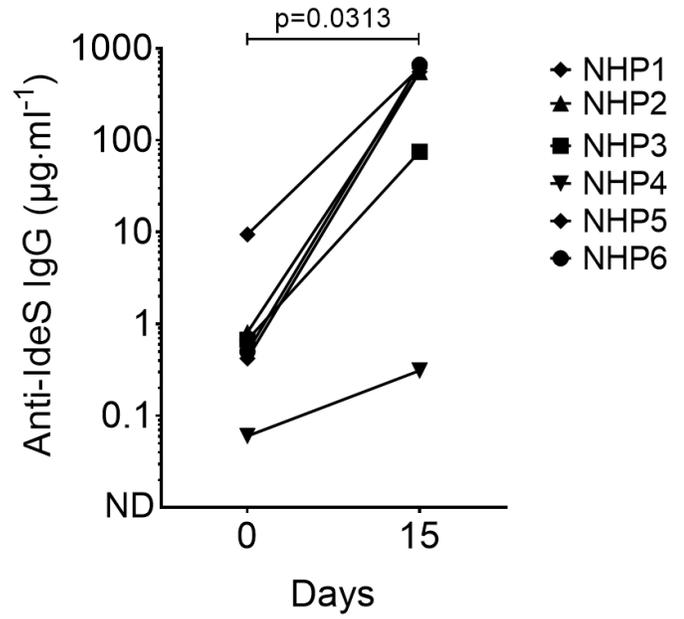
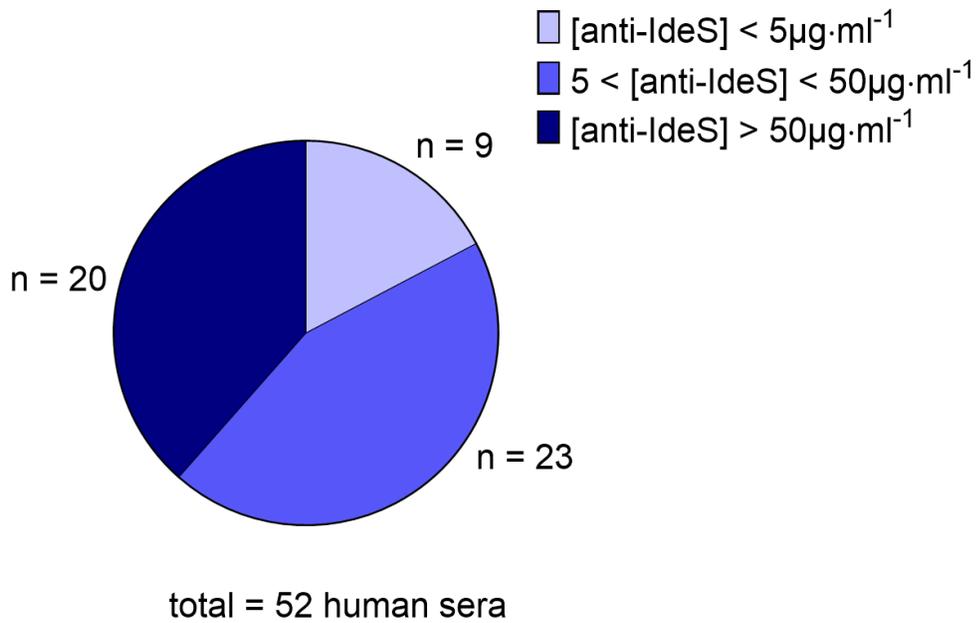
a**b****c****d****e****f****g**

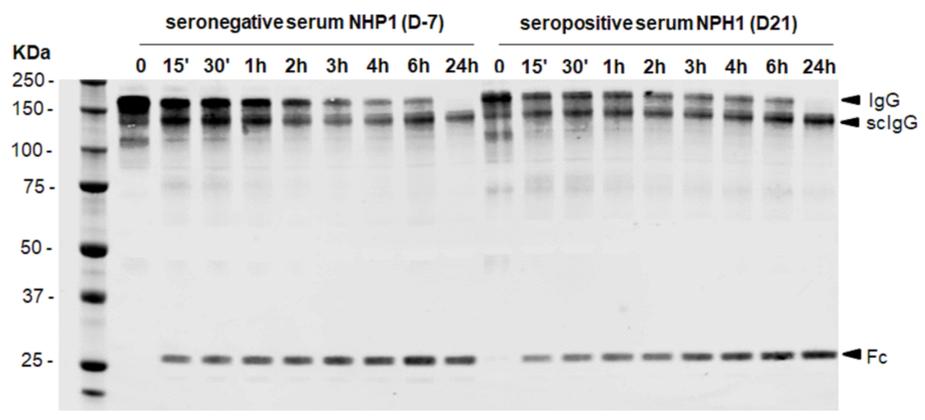
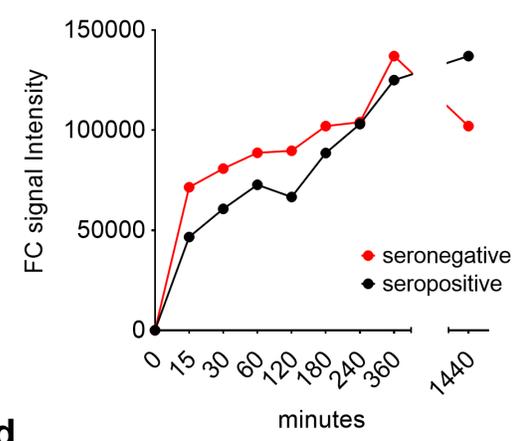
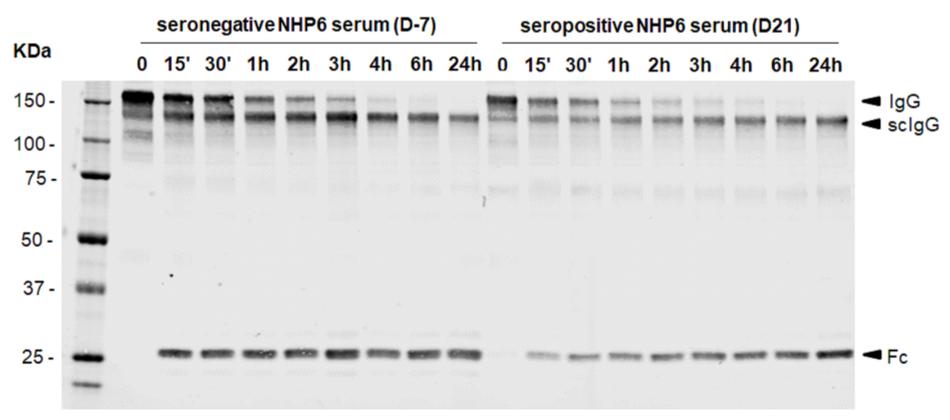


□ NHP1 (control/treated) □ NHP4 (treated/treated)





a**b****c**

a**b****c****d**