

1 **Bispecific Targeting of Conserved Epitopes Enables Pan-Hantavirus Neutralization**

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25

26 **ABSTRACT**

27 Hantaviruses cause renal and cardiopulmonary syndromes with case-fatality rates up to  
28 40%, yet no approved antiviral therapy exists. Antigenic divergence between Old World and  
29 New World hantaviruses has impeded the development of broadly protective antibody thera-  
30 peutics. Here, we isolated neutralizing antibodies from convalescent donors and identified  
31 two lead antibodies targeting distinct conserved epitopes. XA-3H12 potently neutralizes Old  
32 World hantaviruses, whereas XA-044 broadly neutralizes HTNV, SNV and ANDV. Cryo-EM  
33 structures revealed that XA-044 recognizes the apical Gn-Gc interface, whereas XA-3H12  
34 binds the basal region of Gc domain II, defining two non-overlapping conserved neutraliza-  
35 tion sites. Guided by these structures, we engineered a bispecific IgG-scFv that integrates the  
36 breadth and potency of both parental antibodies and achieves broad hantavirus neutralization.  
37 In mouse models, the IgG-scFv blocked entry of diverse hantavirus pseudoviruses *in vivo* and  
38 conferred post-exposure protection against lethal HTNV challenge, establishing a dual-  
39 epitope bispecific antibody candidate for broad hantavirus intervention.

## 40 INTRODUCTION

41 Hantaviruses are enveloped, segmented, negative-sense RNA viruses of the order *Bunya-*  
42 *virales*, broadly classified into Old World and New World lineages according to their geo-  
43 graphic distribution and associated disease manifestations<sup>1-3</sup>. Old World hantaviruses (OWH),  
44 including Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava-  
45 Belgrade virus (DOBV), circulate predominantly in Europe and Asia and cause hemorrhagic  
46 fever with renal syndrome (HFRS), with case-fatality rates ranging from 1% to 15%<sup>4-6</sup>. In  
47 contrast, New World hantaviruses (NWH), such as Andes virus (ANDV) and Sin Nombre vi-  
48 rus (SNV), are endemic to the Americas and cause hantavirus cardiopulmonary syndrome  
49 (HCPS), a severe respiratory disease with fatality rates approaching 40%<sup>4,7,8</sup>. Rodents serve  
50 as the natural reservoirs, and human infection occurs primarily through inhalation of aeroso-  
51 lized virus-contaminated excreta<sup>9</sup>. Despite the substantial morbidity and mortality associated  
52 with hantavirus infections, no FDA-approved antiviral therapies are currently available<sup>6,10</sup>.  
53 Clinical management remains largely supportive, underscoring the urgent need for effective  
54 prophylactic and therapeutic countermeasures.

55 The hantavirus genome comprises three segments—small (S), medium (M), and large  
56 (L)—which encode the nucleocapsid protein (NP), the glycoprotein precursor (GPC), and the  
57 RNA-dependent RNA polymerase (RdRp), respectively<sup>11</sup>. The GPC is processed by host sig-  
58 nal peptidase into two surface glycoproteins, Gn and Gc, which assemble into tetrameric,  
59 square-shaped (Gn/Gc)<sub>4</sub> on the virion surface<sup>12,13</sup>. These glycoproteins mediate viral attach-  
60 ment, endosomal entry, and membrane fusion, and constitute the principal targets of the host  
61 neutralizing antibody response<sup>14-19</sup>. Accordingly, Gn and Gc represent the most attractive an-  
62 tigenic targets for vaccine design and antibody-based therapeutic development.

63 Although antibody-based countermeasures hold considerable promise, developing a  
64 broadly protective therapeutic capable of addressing the extensive antigenic diversity of han-  
65 taviruses remains a major challenge. Previously identified monoclonal antibodies have  
66 demonstrated encouraging activity, but important gaps remain. For example, neutralizing anti-  
67 bodies isolated from convalescent donors protected against NWH, yet their neutralizing ca-  
68 pacity against major HFRS-causing viruses such as HTNV and SEOV was not defined, and  
69 the lack of epitope mapping limited mechanistic understanding<sup>3,18</sup>. More recently, the broadly

70 neutralizing antibody ADI-42898, isolated from a PUUV-infected individual<sup>20,21</sup>, representing  
71 an important conceptual advance for the development of pan-hantavirus antibody counter-  
72 measures. However, ADI-42898 showed relatively limited neutralizing potency against sev-  
73 eral clinically important hantaviruses, including HTNV, and its activity relies on recognition  
74 of a single conserved epitope. Thus, antibody-based therapeutics that achieve broad-spectrum  
75 neutralization by simultaneously targeting two distinct conserved epitopes—and thereby may  
76 reduce the likelihood of viral escape—remain an important unmet need.

77 Here, we address this gap by isolating and characterizing a panel of potent neutralizing  
78 antibodies from four HFRS convalescent donors. From this panel, we identified two lead anti-  
79 bodies with distinct neutralization profiles: XA-3H12, a highly potent neutralizer of the  
80 OWHs HTNV and SEOV, and XA-044, a broadly reactive antibody that neutralizes HTNV as  
81 well as the NWHs SNV and ANDV. Cryo-EM analyses revealed that these antibodies recog-  
82 nize two non-overlapping conserved epitopes on the hantavirus Gn-Gc glycoprotein complex,  
83 providing a structural basis for their neutralizing activities. Guided by these findings, we eval-  
84 uated multiple bispecific antibody formats and identified an IgG-scFv derivative with the  
85 strongest breadth and potency. This bispecific antibody achieved broad-spectrum neutraliza-  
86 tion across OWHs and NWHs, blocked viral entry *in vivo* in a bioluminescent vesicular sto-  
87 matitis virus (VSV)-pseudotyped hantavirus reporter system, and provided robust post-expo-  
88 sure protection in a lethal HTNV mouse challenge model. Together, these findings establish a  
89 dual-epitope bispecific antibody as a promising pan-hantavirus therapeutic candidate and pro-  
90 vide structural guidance for the rational design of next-generation hantavirus vaccines.

91

## 92 **RESULTS**

### 93 **Isolation of cross-reactive antibodies from HFRS convalescent patients**

94 We collected blood samples from four individuals who had recovered from HFRS.  
95 Plasma from all four donors showed strong binding to HTNV and SEOV Gn and Gc antigens  
96 (*Extended Data Fig. 1a,b*). To assess cross-reactive neutralizing activity against both OWH  
97 and NWH, we used recombinant VSV pseudoviruses (rVSV) bearing the Gn-Gc glycopro-  
98 teins of HTNV, SEOV, SNV, and ANDV<sup>22,23</sup>. Donor plasma exhibited broad and potent

99 neutralization of OWH pseudoviruses, whereas neutralization of NWH pseudoviruses was  
100 variable but detectable (**Fig. 1a**).

101 To isolate antigen-specific memory B cells, peripheral blood mononuclear cells  
102 (PBMCs) were sorted using biotinylated HTNV and SEOV Gn and Gc proteins as probes, fol-  
103 lowed by single-cell PCR amplification or single-cell B-cell receptor sequencing (scBCR-seq)  
104 (**Fig. 1b and Extended Data Fig. 1c**). We sorted 384 Gn/Gc-specific single B cells and recov-  
105 ered 323 paired heavy- and light-chain sequences for recombinant IgG expression. An addi-  
106 tional 68 paired sequences were selected for synthesis based on somatic hypermutation  
107 (SHM) rates and complementarity-determining region 3 (CDR3) lengths. Screening of the re-  
108 sulting recombinant antibodies against Gn and Gc from HTNV, SEOV, SNV, and ANDV  
109 identified 117 antibodies reactive with at least one virus (**Extended Data Table 1**). The mean  
110 SHM rates of the heavy and light chains were 7.9% and 5.3%, respectively (**Fig. 1c**), and the  
111 mean CDR3 lengths were 18 and 10 amino acids, respectively (**Fig. 1d**). Among these anti-  
112 bodies, 9 Gn-directed and 21 Gc-directed antibodies displayed cross-reactivity across multiple  
113 viruses. Gn cross-reactive antibodies were largely restricted to OWH, whereas Gc-directed  
114 antibodies exhibited broader reactivity, including strong binding to NWH Gc (**Fig. 1e**). Col-  
115 lectively, these data demonstrate that cross-reactive antibodies targeting diverse hantaviruses  
116 exist in HFRS convalescent donors.

117

#### 118 **Identification of NAbs targeting distinct hantavirus Gn/Gc neutralization sites**

119 We next screened Gn/Gc-specific antibodies for binding to the Gn head domain and Gc  
120 ectodomain (**Fig. 2a**), and for neutralizing activity against rVSV-HTNV (**Extended Data Ta-**  
121 **ble 1**). Fifteen NAbs, including 10 derived from single-cell PCR and 5 from scBCR-seq, were  
122 selected for further characterization (**Extended Data Fig. 2a**). Binding was assessed against  
123 isolated Gn head, Gc ectodomain, and a heterodimeric Gn<sup>H</sup>Gc construct that recapitulates the  
124 native quaternary architecture of hantavirus glycoproteins (**Fig. 2b**)<sup>12</sup>. Most antibodies pref-  
125 erentially recognized OWH antigens, with limited binding to NWH glycoproteins (**Fig. 2c**  
126 **and Extended Data Fig. 2b, c**). Several antibodies, including XA-1A5, XA-1D4, XA-044,  
127 and XA-060, bound weakly to isolated Gn or Gc but recognized Gn<sup>H</sup>Gc, indicating

128 conformation-dependent epitopes. Notably, XA-044 was the only antibody that strongly  
129 bound NWH Gn<sup>H</sup>Gc, recognizing HTNV, SNV, and ANDV, but not SEOV.

130 Neutralization assays against OWH and NWH pseudoviruses showed that 12 antibodies  
131 potently neutralized rVSV-HTNV, with IC<sub>50</sub> values below 0.01 µg/mL, whereas three showed  
132 moderate activity. Five antibodies—XA-3A8, XA-3H12, XA-4E4, XA-4G11, and XA-058—  
133 cross-neutralized rVSV-HTNV and rVSV-SEOV (*Fig. 2d and Extended Data Fig. 2d*). Con-  
134 sistent with its binding profile, XA-044 potently neutralized HTNV, SNV, and ANDV pseu-  
135 doviruses, but not SEOV. Five antibodies also neutralized authentic HTNV, among which  
136 XA-3H12 and XA-044 were the most potent, with IC<sub>50</sub> values of 0.034 and 0.013 µg/mL, re-  
137 spectively (*Fig. 2d and Extended Data Fig. 2e*).

138 To map the antigenic landscape, we performed Biolayer Interferometry (BLI)-based  
139 competition assays using HTNV glycoproteins. The 15 NAbs segregated into five antigenic  
140 sites, A1, A2, B, C, and D (*Fig. 2e and Extended Data Fig. 3*). Site A1, targeted by XA-044,  
141 represented a conserved epitope shared by HTNV, SNV, and ANDV, whereas site C targeted  
142 by XA-3H12 defined a major OWH neutralizing site. Sites B and D were primarily associated  
143 with OWH Gn- and Gc-directed antibodies, respectively. These data reveal multiple neutraliz-  
144 ing vulnerabilities on hantavirus Gn<sup>H</sup>Gc with distinct breadth profiles.

145

#### 146 **Structural basis of broad neutralization by XA-044 and XA-3H12**

147 Having identified XA-044 and XA-3H12 as two of the most potent NAbs targeting dis-  
148 tinct antigenic sites with complementary breadth profiles—site A1 and site C, respectively—  
149 we next sought to define the structural basis of their recognition and neutralization. We there-  
150 fore determined the structures of XA-044 Fab bound to SNV Gn<sup>H</sup>Gc and XA-3H12 Fab  
151 bound to HTNV Gn<sup>H</sup>Gc (*Extended Data Table 2*). Superposition onto the ANDV Gn-Gc di-  
152 mer (PDB: 9P3X)<sup>13</sup> showed that XA-044 targets the apical Gn-Gc interface, whereas XA-  
153 3H12 binds the basal region of Gc domain II (*Fig. 3a*). XA-044 buries >720 Å<sup>2</sup> of surface  
154 area through shape complementarity and seven hydrogen bonds, using CDR L1-2 and H3 to  
155 contact SNV Gn residues 73-79 and Gc residues 90-92, 129-130, 91-93, 110-115, 129 and  
156 131 (*Fig. 3b,d*). Its epitope partially overlaps that of ADI-65534<sup>13,21</sup>, but is shifted toward the  
157 Gc fusion-loop region while retaining Gn capping-loop contacts (*Extended Data Fig. 4a*). In

158 contrast, XA-3H12 buries  $>1025 \text{ \AA}^2$  on HTNV Gc alone through shape complementarity, hy-  
159 drogen bonds and salt bridges mediated by CDR L1, L3, H1-3 and FR3, contacting residues  
160 67-72, 147-150, 152, 194-195, 216-219, 225-238 (except 227 and 229), and 288-289; the  
161 heavy chain forms eight hydrogen bonds and two salt bridges, and the light chain forms three  
162 hydrogen bonds (**Fig. 3e,g**). Notably, N-glycosylation protruded from N328 of HTNV Gn  
163 may also stabilize XA-3H12 binding (**Extended Data Fig. 4b**).

164 Conservation analysis showed that 9/13 XA-044 epitope residues on SNV Gc are fully  
165 conserved and 13/13 are  $>70\%$  conserved across OWHs and NWHs, whereas 6/7 Gn residues  
166 are  $>50\%$  conserved; for XA-3H12, 10/31 epitope residues on HTNV Gc are fully conserved  
167 and 25/31 are  $>50\%$  conserved (**Fig. 3h,i**). Structural superposition onto the ANDV Gn-Gc  
168 tetramer (PDB: 9P3Y)<sup>13</sup> revealed no clash within a single tetramer, but in the tetramer-dimer  
169 interface XA-044 shows minor or no clash with neighboring Gc, whereas XA-3H12 exhibits a  
170 major clash with neighboring Gn (**Fig. 3j and Extended Data Fig. 4c,d**). Together, these  
171 structures identify two spatially distinct and conserved neutralizing epitopes on the hantavirus  
172 Gn-Gc complex that underlie the complementary neutralization profiles of XA-044 and XA-  
173 3H12. The non-overlapping binding modes, together with their distinct breadth and potency,  
174 provided a structural rationale for combining these antibodies and for engineering bispecific  
175 formats capable of simultaneously engaging both neutralizing sites.

176

### 177 **Bispecific antibody strategies achieve pan-hantavirus neutralization**

178 Guided by the structural demonstration that XA-044 and XA-3H12 recognize spatially  
179 distinct and conserved neutralizing epitopes, and by their complementary neutralization  
180 breadth, we next asked whether co-targeting these two sites could enhance potency and ex-  
181 pand hantavirus coverage. We therefore performed BLI analyses to characterize the binding  
182 profiles of XA-044 and XA-3H12 against representative OWH and NWH Gn<sup>H</sup>Gc antigens.  
183 XA-3H12 bound HTNV, SEOV, and SNV Gn<sup>H</sup>Gc with picomolar to subpicomolar affinities,  
184 whereas XA-044 bound ANDV Gn<sup>H</sup>Gc with subpicomolar affinity ( $K_D < 1 \text{ pM}$ ) and also  
185 showed high-affinity binding to HTNV and SNV Gn<sup>H</sup>Gc, but not SEOV (**Fig. 4a and Ex-**  
186 **tended Data Fig. 5a**). Consistent with the structural analysis, BLI-based competition assays  
187 confirmed that XA-044 and XA-3H12 can simultaneously engage Gn<sup>H</sup>Gc without detectable

188 competition, supporting their combined use (**Fig. 4b**). We next assessed whether these two  
189 antibodies act synergistically in neutralization. In a checkerboard assay spanning concentra-  
190 tions around their individual IC<sub>50</sub> values, the XA-044/XA-3H12 cocktail achieved 91.67%  
191 neutralization at the combined IC<sub>50</sub> concentration, exceeding the 75% expected from simple  
192 additivity (**Fig. 4c**). These results indicate that co-targeting both neutralization sites produces  
193 synergistic enhancement of neutralization potency.

194 To identify an optimal combinatorial format, we evaluated five designs: a two-antibody  
195 cocktail, Knob-into-Hole, CrossMab, Fab-scFv, and IgG-scFv. All formats potently neutral-  
196 ized HTNV, with the cocktail, Fab-scFv, and IgG-scFv achieving IC<sub>50</sub> values below 1 ng/mL  
197 (**Fig. 4d and Extended Data Fig. 5b**). However, Knob-into-Hole, CrossMab, and Fab-scFv  
198 showed reduced activity against NWH pseudoviruses, including SNV, ANDV, JUQV, and  
199 LNV. By contrast, the cocktail and IgG-scFv preserved the breadth and potency of both pa-  
200 rental antibodies, with IC<sub>50</sub> values comparable to those of XA-044 or XA-3H12 alone (**Fig.**  
201 **4d and Extended Data Fig. 5b**). Consistently, cell-surface staining of cells expressing full-  
202 length viral glycoproteins showed that IgG-scFv combined the binding breadth of both paren-  
203 tal antibodies. Whereas XA-3H12 and XA-044 each displayed more restricted binding pro-  
204 files, IgG-scFv recognized all tested viruses bound by either antibody, including the OWHs  
205 HTNV, SEOV, DOBV, and PUUV, as well as the NWHs SNV, ANDV, LNV, JUQV, and  
206 CHOV (**Fig. 4e,f and Extended Data Fig. 5c**). Together, these *in vitro* binding and neutrali-  
207 zation data demonstrate that the structurally guided IgG-scFv design successfully integrates  
208 the complementary breadth and potency of XA-044 and XA-3H12 into a single bispecific  
209 molecule. Thus, we advanced the IgG-scFv for subsequent *in vivo* evaluation, with the paren-  
210 tal-antibody cocktail included as a benchmark comparator.

211

## 212 **A pseudovirus reporter system enables *in vivo* evaluation of hantavirus entry blockade**

213 To evaluate hantavirus NAb *in vivo* under Animal Biosafety Level (ABSL)-2 condi-  
214 tions, we generated four luciferase-expressing hantavirus pseudoviruses: rVSV-HTNV-Luc,  
215 rVSV-SEOV-Luc, rVSV-SNV-Luc, and rVSV-ANDV-Luc (**Fig. 5a**). After intraperitoneal  
216 injection into BALB/c mice, bioluminescence imaging over 9 hours showed that luciferase  
217 signals became detectable at 2 hours post-infection, increased progressively from 2 to 6 hours,

218 and then plateaued between 6 and 9 hours (**Fig. 5b and Extended Data Fig. 6a**). Three-di-  
219 mensional optical tomography (**Fig. 5c and Extended Data Fig. 6b**) and immunohistochemis-  
220 try (**Fig. 5d and Extended Data Fig. 6c**) further identified the lungs and intestines as the prin-  
221 cipal sites of pseudovirus accumulation. These results establish an ABSL-2-compatible *in*  
222 *vivo* reporter platform for tracking hantavirus pseudovirus entry and define a suitable time  
223 window for assessing antibody-mediated entry blockade.

224 To validate this system, pseudoviruses were pre-incubated with antibodies for 1 hour at  
225 37°C before intraperitoneal administration (**Fig. 5e**). At 6 hours post-infection, mice receiving  
226 isotype control antibody showed robust bioluminescence, whereas treatment with XA-3H12,  
227 XA-044, the XA-3H12/XA-044 cocktail, or IgG-scFv markedly reduced HTNV pseudovirus  
228 signals (**Fig. 5f and Extended Data Fig. 7a**). For SEOV pseudovirus, efficient entry blockade  
229 was observed with XA-3H12, the cocktail, and IgG-scFv, whereas XA-044 alone showed lim-  
230 ited activity (**Fig. 5g and Extended Data Fig. 7a**). Conversely, XA-044, the cocktail, and  
231 IgG-scFv potently inhibited SNV and ANDV pseudovirus entry, while XA-3H12 alone was  
232 ineffective (**Fig. 5h,i and Extended Data Fig. 7a**). Thus, the *in vivo* entry-blockade profiles  
233 closely matched the *in vitro* neutralization breadth of each antibody, validating this pseudo-  
234 virus reporter system as a robust platform for evaluating hantavirus NAb *in vivo*.

235

### 236 **Bispecific strategies confer broad hantavirus entry blockade *in vivo***

237 To evaluate the prophylactic efficacy of these antibodies *in vivo*, we administered indi-  
238 vidual mAbs, the XA-044/XA-3H12 cocktail, or the bispecific IgG-scFv intraperitoneally to  
239 BALB/c mice 18 hours before challenge with luciferase-expressing hantavirus pseudoviruses.  
240 Viral entry was assessed by bioluminescence imaging 6 hours after pseudovirus challenge  
241 (**Fig. 6a**). Compared with isotype-control-treated mice, all antibody-treated groups showed  
242 markedly reduced HTNV pseudovirus signals, indicating effective blockade of HTNV entry  
243 by XA-044, XA-3H12, the cocktail, and IgG-scFv (**Fig. 6b and Extended Data Fig. 7b**).  
244 Consistent with their *in vitro* neutralization profiles and the pre-incubation blockade results,  
245 XA-044 conferred limited protection against SEOV pseudovirus, whereas XA-3H12 failed to  
246 protect against SNV and ANDV pseudoviruses. In contrast, mice pre-treated with either the  
247 XA-044/XA-3H12 cocktail or IgG-scFv exhibited markedly reduced luminescence across all

248 four pseudovirus challenges (**Fig. 6b,e and Extended Data Fig. 7b**). These results demon-  
249 strate that both the antibody cocktail and the bispecific IgG-scFv provide broad prophylactic  
250 protection against entry of diverse hantavirus pseudoviruses, spanning both OWHs and  
251 NWHs.

### 253 **Post-exposure antibody therapy protects mice from lethal HTNV challenge**

254 We next evaluated the therapeutic efficacy of the XA-044/XA-3H12 cocktail and the  
255 bispecific IgG-scFv in a lethal authentic HTNV infection model in suckling mice. Given the  
256 clinical relevance of post-exposure intervention, antibodies were administered 24 h after  
257 HTNV challenge (**Fig. 6f**). In the isotype-control group, mortality began on day 15, and all  
258 animals succumbed to infection by day 18 post-infection. By contrast, both the XA-044/XA-  
259 3H12 cocktail and the bispecific IgG-scFv conferred substantial protection, with only 1 and 2  
260 deaths observed in the cocktail- and IgG-scFv-treated groups, respectively (**Fig. 6g**). Con-  
261 sistent with these survival outcomes, isotype-control-treated mice exhibited weight stagnation  
262 or loss beginning around day 14 post-infection, whereas antibody-treated mice continued to  
263 gain weight throughout the observation period (**Fig. 6g**). Quantification of viral RNA by  
264 qRT-PCR at the endpoint further showed that HTNV RNA was undetectable or markedly re-  
265 duced in the brain, lungs, spleen, and kidneys of antibody-treated mice, indicating that post-  
266 exposure antibody administration effectively restricted systemic viral dissemination (**Fig. 6h**).  
267 Collectively, these findings demonstrate that both the XA-044/XA-3H12 cocktail and the  
268 bispecific IgG-scFv provide potent post-exposure protection against lethal HTNV infection *in*  
269 *vivo*.

### 271 **DISCUSSION**

272 Hantaviruses remain a persistent and significant zoonotic threat, as illustrated by the re-  
273 cent Andes virus outbreak linked to a South American cruise ship<sup>24</sup>. Their broad rodent reser-  
274 voirs, recurrent spillover, and—in the case of Andes virus—capacity for human-to-human  
275 transmission underscore the need for countermeasures that can protect against diverse hanta-  
276 viruses, including those that may emerge in future outbreaks. However, no approved specific  
277 therapeutics or broadly protective vaccines are currently available. Antibody development has

278 been constrained by the antigenic diversity of the Gn-Gc glycoprotein complex, with most  
279 neutralizing antibodies exhibiting either lineage-restricted activity or limited broad-spectrum  
280 potency when targeting a single epitope. In this study, we establish a dual-epitope bispecific  
281 strategy to overcome these limitations and broaden hantavirus antibody coverage. By simulta-  
282 neously engaging two distinct conserved neutralization sites recognized by XA-044 and XA-  
283 3H12 within an IgG-scFv architecture, we achieve broad neutralization against representative  
284 OWHs and NWHs, conferring robust *in vivo* protection across diverse pseudovirus and lethal  
285 HTNV challenge models.

286 Cryo-EM structures reveal the basis of broad neutralization against both OWHs and  
287 NWHs. XA-044 recognizes a conserved epitope at the apical Gn-Gc interface, with key con-  
288 tacts extending into the Gc fusion-loop region<sup>12</sup>. Although this epitope includes a relatively  
289 less conserved Gn capping loop, its broader footprint on the highly conserved apical Gc sur-  
290 face likely underlies the breadth of XA-044, and suggests a mechanism of fusion blockade  
291 similar to that proposed for ADI-65534 and ADI-42898<sup>13,21</sup>. XA-3H12, in contrast, binds the  
292 basal region of Gc domain II and likely disrupts Gn-Gc lattice assembly on the virion surface.  
293 Thus, XA-044 and XA-3H12 define two spatially separated but conserved vulnerabilities on  
294 the hantavirus glycoprotein complex.

295 The structural and functional data support a combinatorial strategy for achieving broad-  
296 spectrum hantavirus protection. Although individual antibodies with cross-reactive activity  
297 have been described<sup>20,21</sup>, the antigenic divergence between OWHs and NWHs makes it diffi-  
298 cult for a single specificity to combine maximal potency and breadth. In this context, XA-044  
299 and XA-3H12 provide a rational two-antibody solution. Competition-binding experiments  
300 demonstrated that the two antibodies recognize non-overlapping epitopes on the Gn-Gc com-  
301 plex and can bind antigen simultaneously, while neutralization assays showed that their com-  
302 bined use produces synergistic activity. These findings provide a mechanistic rationale for  
303 dual targeting: simultaneous engagement of two conserved neutralizing sites can expand  
304 broad-spectrum coverage while potentially increasing the genetic barrier to escape. To trans-  
305 late this concept into a single-molecule format, we evaluated five bispecific antibody designs  
306 and found that only the IgG-scFv format matched or exceeded the cocktail. A plausible expla-  
307 nation is architectural: the longer and more flexible IgG-scFv format may better

308 accommodate the distance between the XA-044 and XA-3H12 epitopes, allowing simultane-  
309 ous engagement on the same virion or neighboring glycoprotein complexes. By contrast,  
310 more compact formats, including knob-into-hole, CrossMab, and Fab-scFv, may position the  
311 two binding arms too closely or impose geometric constraints that limit productive dual en-  
312 gagement. Importantly, with efficacy comparable to the cocktail *in vitro* and *in vivo*, the IgG-  
313 scFv format offers practical advantages as a single therapeutic product, including simplified  
314 manufacturing, release testing, dosing, and clinical development relative to a two-component  
315 cocktail. More broadly, bispecific targeting of two conserved but distinct epitopes may pro-  
316 vide a generalizable strategy for developing antibody countermeasures against antigenically  
317 diverse bunyaviruses.

318 A major practical barrier in hantavirus countermeasure development is the restricted ac-  
319 cess, high cost and low throughput of authentic-virus studies under high-containment condi-  
320 tions. For instance, HTNV and SEOV generally require ABSL-3, whereas highly pathogenic  
321 NWHs such as ANDV and SNV require ABSL-4. To address this limitation, we established a  
322 ABSL-2-compatible bioluminescent pseudovirus challenge model bearing hantavirus Gn-Gc  
323 glycoproteins, enabling rapid and quantitative *in vivo* assessment of neutralizing antibodies  
324 across multiple hantaviruses. The concordance between this model and the authentic HTNV  
325 lethal challenge—both identifying the cocktail and IgG-scFv as protective and revealing the  
326 limitations of individual antibodies—supports its utility as a preclinical prioritization tool.  
327 Although this system cannot fully replace authentic-virus protection studies, it can serve as an  
328 efficient prescreening step to prioritize candidates before costly and labor-intensive high-con-  
329 tainment experiments. This platform should also facilitate evaluation of both passive anti-  
330 body-based protection and active vaccine-induced neutralizing responses against panels of an-  
331 tigenically diverse hantaviruses.

332 This study has several limitations. First, although protection against authentic HTNV  
333 was demonstrated, live-virus validation against BSL-4-restricted ANDV and SNV remains  
334 essential but was beyond the scope of the present study due to containment and strain-access  
335 limitations. Second, further studies using serial passage or deep mutational scanning will be  
336 needed to define potential escape mutations for both the antibody cocktail and the IgG-scFv  
337 bispecific antibody. Third, the *in vivo* pseudovirus model does not capture post-entry steps of

338 viral replication, pathogenesis, or Fc-mediated antibody functions. Finally, the neonatal  
339 mouse HTNV challenge model does not fully recapitulate adult immunity or human hanta-  
340 virus disease, therefore the 24 h post-exposure efficacy observed in this model has limited  
341 clinical relevance; the therapeutic window, optimal dosing, and durability of protection  
342 should be further evaluated in more disease-relevant models, such as non-human primates.  
343 Nonetheless, the ability of XA-044 and XA-3H12 to simultaneously target two conserved  
344 sites of vulnerability on the Gn-Gc complex supports a dual-specificity strategy for both anti-  
345 body therapy and rational pan-hantavirus vaccine design.

346

## 347 **METHODS**

### 348 **Human subjects and PBMC isolation**

349 Convalescent blood samples were collected from four HFRS donors admitted by Tangdu  
350 hospital in 2023-2024. PBMCs and matched plasma samples were obtained from convales-  
351 cent patients more than 6 months after discharge. The participants gave written informed con-  
352 sent to participate in this study. This study was approved by the Ethics Review Committee of  
353 Tangdu Hospital (IEC of Institution for National Drug Clinical Trials, Tangdu Hospital,  
354 Fourth Military Medical University, approval # 202409-14 and K202410-35) and Ethics Re-  
355 view Committee of Westlake University (approval # 20240423LDP001).

356 Peripheral blood samples were obtained from donors in EDTA-coated collection tubes  
357 and processed immediately for PBMC isolation using lymphocyte separation tubes  
358 (DAKEWE, Cat # 7922112). Blood samples were centrifuged at  $400 \times g$  for 20 min to sepa-  
359 rate plasma and cellular components. The plasma fraction was harvested and preserved at  
360  $-80^\circ\text{C}$  for subsequent analyses. The remaining blood cells were diluted with an equal volume  
361 of D-PBS (Servicebio, Cat # G4200) and subjected to density-gradient centrifugation. Mono-  
362 nuclear cells enriched at the interphase were carefully collected and stored at  $-80^\circ\text{C}$  until fur-  
363 ther use.

364

### 365 **Recombinant Protein Expression, Purification, and Biotinylation**

366 Codon-optimized sequences encoding the Gn head domain (1,062 bp) and Gc ectodo-  
367 main (1,347 bp) consensus sequences of HTNV and SEOV<sup>25</sup> were individually cloned into

368 the *Drosophila* S2 expression vector pMT/BiP with a C-terminal Avi-tag and 6×His-tag. The  
369 Gn head domain (1,071 bp) and Gc ectodomain (1,341 bp) sequences of SNV<sup>26</sup>, and the Gn  
370 head domain (1,068 bp) and Gc ectodomain (1,350 bp) sequences of ANDV<sup>27</sup> were individu-  
371 ally cloned into the *Drosophila* S2 expression vector pMT/BiP with a C-terminal 6×His-tag.  
372 For the generation of heterodimeric Gn<sup>H</sup>Gc constructs from HTNV, SEOV, SNV, and ANDV,  
373 the Gn head domain and Gc ectodomain were linked by the amino acid sequence  
374 GGSGLVPRGSGGGSGGGSWSHPQFEKGGGTGGGTLVPRGSGTGG as previously de-  
375 scribed<sup>12</sup>, and the resulting constructs were individually cloned into pMT/BiP with a C-termi-  
376 nal 6×His-tag. Recombinant plasmids were transfected into *Drosophila* S2 cells (Invitrogen,  
377 Cat # K2780-01), and stable cell lines were generated by selection with blasticidin (25 µg/ml;  
378 Beyotime, Cat # ST018). Protein expression was induced with 10 µM CdCl<sub>2</sub> (Macklin, Cat #  
379 C805627) for 5 days. Culture supernatants were collected, filtered through a 0.22-µm mem-  
380 brane, and buffer-exchanged using a 10-kDa MWCO VIVAFLOW 200 module (Sartorius,  
381 Cat # VF20H0) to remove residual CdCl<sub>2</sub>. Recombinant proteins were purified by Ni<sup>2+</sup> affini-  
382 ty chromatography (SunResin, Cat # A453201) using an ÄKTA system and subsequently bio-  
383 tynylated through the Avi-tag using a Biotin Labeling Kit (Beyotime, Cat # P0630S).

384

### 385 **Isolation and Sorting of Antigen-Specific B Cells**

386 B cells were enriched from PBMCs using a human pan-B cell isolation kit (BioLegend,  
387 Cat # 480082) and stained with Zombie Aqua viability dye (BioLegend, Cat # 423102), anti-  
388 human CD14-BV605 (BioLegend, Cat # 301834), anti-human CD16-BV785 (BioLegend, Cat  
389 # 302046), anti-human CD3-PE-Cy5 (BioLegend, Cat # 300310), anti-human CD19-APC-  
390 Cy7 (BioLegend, Cat # 363010), anti-human IgD-FITC (BioLegend, Cat # 348206), and anti-  
391 human IgM-PerCP-Cy5.5 (BD Biosciences, Cat # 561285) for 30 min at 4 °C in the dark. To  
392 identify HTNV- and SEOV-specific B cells, biotinylated Gn or Gc proteins were precom-  
393 plexed with PE-streptavidin (BioLegend, Cat # 405204) or APC-streptavidin (BioLegend, Cat  
394 # 405207) to generate tetrameric probes, followed by incubation with enriched B cells. Cells  
395 were analyzed and sorted on a CytoFLEX SRT flow cytometer (Beckman Coulter). Antigen-  
396 specific B cells were defined as CD14<sup>-</sup>CD16<sup>-</sup>CD3<sup>-</sup>CD19<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup>PE<sup>+</sup> or  
397 CD14<sup>-</sup>CD16<sup>-</sup>CD3<sup>-</sup>CD19<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup>APC<sup>+</sup> populations.

398 For antibody gene amplification and cloning, single antigen-specific B cells were sorted  
399 into 96-well PCR plates containing 20  $\mu$ L lysis buffer per well [2.5  $\mu$ L 10 $\times$  RT buffer, 2.5  $\mu$ L  
400 5 $\times$  gDNA wiper mix (Vazyme, Cat # R312), 0.0625  $\mu$ L IGEPAL (Sigma-Aldrich, Cat #  
401 I8896), 0.5  $\mu$ L carrier RNA (Sangon Biotech, Cat # B518271-0100), and 14.4375  $\mu$ L nucle-  
402 ase-free water]. Plates were briefly centrifuged and stored at  $-80^{\circ}\text{C}$  until use. For single-cell  
403 transcriptome and BCR sequencing, antigen-specific B cells were sorted into D-PBS contain-  
404 ing 2% FBS, counted, and processed for downstream sequencing analyses.

405

#### 406 **Single B Cell PCR**

407 Single B-cell antibody gene amplification and cloning were performed as previously de-  
408 scribed<sup>28</sup>. Briefly, cDNA was synthesized from individually sorted B cells using the HiScript  
409 III First-Strand cDNA Synthesis Kit (Vazyme, Cat # R312). Immunoglobulin heavy-chain  
410 (HC) and light-chain (LC) variable regions were amplified by two rounds of PCR. The first  
411 round used primers targeting the 5' leader and immunoglobulin constant regions to amplify  
412 multiple Ig isotypes, followed by nested PCR for specific amplification of HC and LC varia-  
413 ble regions and incorporation of homologous sequences compatible with expression vectors.  
414 PCR products were analyzed on 1% agarose gels, and fragments of approximately 500 bp  
415 (HC) and 450 bp (LC) were purified, linearized, and used for small-scale antibody expression.

416

#### 417 **Single-cell BCR sequencing and antibody gene screening**

418 Antigen-specific memory B cells were sorted and loaded onto a Chromium Next GEM  
419 Chip (10x Genomics). Single-cell capture, lysis, and first-strand cDNA synthesis were per-  
420 formed using the Chromium Single Cell 5' Library & Gel Bead Kit (10x Genomics) accord-  
421 ing to the manufacturer's instructions. Gene expression and V(D)J libraries were generated  
422 using the Chromium Single Cell V(D)J Reagent Kits (10x Genomics). Libraries were se-  
423 quenced on an Illumina NovaSeq X Plus platform in paired-end 150-bp mode.

424 Single-cell BCR sequencing data were assembled and initially annotated using the V(D)J  
425 module of Cell Ranger (v7.2), with the GRCh38 Human V(D)J reference dataset (GRCh38-  
426 alts-ensembl-7.1.0) downloaded from 10x Genomics. Among a total of 1,959 cells, 1,894  
427 cells contained productive V-J spanning pairs. Antibody clonal lineage identification was

428 performed using an optimized Change-O workflow. Human IG reference sequences from the  
429 IMGT database were used for IgBLAST annotation and germline reconstruction, while Cell  
430 Ranger V(D)J annotation results were additionally incorporated for single-cell BCR datasets.

431 To determine the clonal clustering threshold, nearest-neighbor distances between produc-  
432 tive BCR heavy-chain sequences were calculated using the `distToNearest` function in the  
433 shazam package. Junction nucleotide sequences sharing identical V- and J-gene assignments  
434 were compared using normalized Hamming distances. The resulting nearest-neighbor distance  
435 distribution typically exhibits a bimodal pattern, representing intra-clonal diversification  
436 driven by SHM and inter-clonal sequence diversity, respectively. A Gaussian mixture model  
437 (GMM) was fitted to the nearest-neighbor distance distribution using the `findThreshold`  
438 function with the `gmm` method. The threshold separating clonally related and unrelated se-  
439 quences was defined as the intersection between the two inferred Gaussian components. This  
440 threshold (0.285103) was subsequently used for clone assignment in downstream repertoire  
441 analyses.

442 Candidate antibodies for downstream characterization were selected from all identified  
443 BCR repertoires according to the following criteria: (i) complete paired heavy- and light-  
444 chain sequences detected within the same single cell; (ii) SHM rate greater than 3%; (iii)  
445 heavy-chain CDRH3 length greater than 17 amino acids; and (iv) light-chain CDRL3 length  
446 greater than 8 amino acids.

447

#### 448 **Expression and purification of mAbs**

449 For antibodies derived from single B-cell PCR, purified PCR products were assembled  
450 into linear human IgG expression cassettes by overlapping PCR as previously described<sup>2</sup>. For  
451 antibodies identified by single-cell BCR sequencing, heavy- and light-chain variable genes  
452 were synthesized and cloned into mammalian expression vectors. Expression cassettes or  
453 plasmids were transiently transfected into Expi293F cells using PEI MAX (Polysciences).  
454 Culture supernatants containing recombinant IgGs were harvested for antibody quantification  
455 and preliminary ELISA screening. For pseudovirus neutralization assays, antibody plasmids  
456 were transfected into 15 mL Expi293F cultures, and antibodies were harvested after 5 days  
457 and purified using protein A magnetic beads (GenScript, Cat # L00695).

458 For large-scale antibody production, heavy- and light-chain plasmids were co-transfected  
459 into Expi293F cells at a 1:1.5 ratio using PEI MAX. Supernatants were collected 5 days post-  
460 transfection, clarified by centrifugation, filtered through a 0.22- $\mu$ m membrane, and purified  
461 by protein A affinity chromatography (SunResin, Cat # A4093101) on an ÄKTA system.  
462 Bound antibodies were eluted with 100 mM glycine (pH 2.5), immediately neutralized with  
463 1/10 volume of 1 M Tris-HCl, and buffer-exchanged into PBS.

464

#### 465 **ELISA**

466 For preliminary screening, 96-well high-binding ELISA plates were coated overnight at  
467 4 °C with glycoproteins (1  $\mu$ g/mL). Plates were washed three times with PBST (0.1% Tween-  
468 20 in D-PBS) and blocked with 3% BSA in PBST for 2 h at 37 °C. Recombinant IgG-contain-  
469 ing supernatants (100  $\mu$ L/well) were added and incubated for 2 h at 37 °C. After five washes,  
470 plates were incubated with HRP-conjugated anti-human IgG antibody (Promega, Cat #  
471 W4031; 1:5,000 dilution) for 1 h at 37 °C. Plates were washed five times, developed with  
472 TMB substrate (Beyotime, Cat # P0209) for 10 min at room temperature in the dark, and the  
473 reaction was terminated with 50  $\mu$ L of 1 M sulfuric acid. Absorbance was measured at 450  
474 nm.

475 For quantitative binding analysis, purified antibodies were serially diluted 3-fold from an  
476 initial concentration of 10  $\mu$ g/mL and added to antigen-coated plates prepared as described  
477 above. Incubation, washing, and detection procedures were performed under identical condi-  
478 tions. All assays were performed in duplicate.

479

#### 480 **Generation of pseudotyped VSVs**

481 All pseudotyped viruses were generated using pLVX plasmids encoding the M segment  
482 of HTNV<sup>29</sup>, SEOV<sup>30</sup>, SNV<sup>26</sup>, ANDV<sup>27</sup>, LNV<sup>31</sup>, or JUQV<sup>32</sup>. HEK293T cells were seeded in  
483 10-cm dishes and transfected 12 h later with M-segment expression plasmids (12  $\mu$ g),  
484 psPAX2 (9  $\mu$ g), and pMD2.G (3  $\mu$ g) using PEI MAX. At 12 h post-transfection, the medium  
485 was replaced with fresh culture medium. Lentivirus-containing supernatants were harvested  
486 48 h later and stored at -80 °C. Stable HEK293T cell lines expressing HTNV M, SEOV M,  
487 SNV M, ANDV M, LNV M, or JUQV M were generated by lentiviral transduction followed

488 by puromycin selection.

489 For rVSV production, all pseudotyped viruses were produced according to previously de-  
490 scribed protocols<sup>33</sup>. Stable M-expressing HEK293T cells were seeded in 10-cm dishes and  
491 infected 12 h later with rVSV-GFP or rVSV-luc for 1 h at 37 °C. Cells were washed five times  
492 with PBS and maintained in Opti-MEM containing 2% FBS. Supernatants containing rVSV-  
493 HTNV, rVSV-SEOV, rVSV-SNV, rVSV-ANDV, rVSV-LNV, or rVSV-JUQV were collected  
494 24-36 h post-infection, clarified by centrifugation, filtered through a 0.45- $\mu$ m membrane, and  
495 stored at -80 °C until use.

496

#### 497 **Pseudovirus neutralization assay**

498 Neutralization activity was evaluated using rVSV-based hantavirus pseudoviruses ex-  
499 pressing GFP, including rVSV-HTNV, rVSV-SEOV, rVSV-SNV, rVSV-ANDV, rVSV-LNV,  
500 and rVSV-JUQV. Vero cells were seeded in 96-well plates at  $5 \times 10^3$  cells per well one day  
501 before infection. Antibodies were subjected to 3-fold serial dilution in complete medium and  
502 pre-incubated with pseudoviruses for 1 h at 37 °C. The antibody-virus mixtures were subse-  
503 quently transferred onto Vero cells and incubated for an additional 1 h at 37 °C to facilitate  
504 viral entry. Following incubation, cells were washed to remove unbound virus, and fresh com-  
505 plete medium was added. Infection was assessed 24 h later by detection of GFP-positive cells  
506 using fluorescence microscopy or an Operetta CLS high-content imaging system (Perkin  
507 Elmer, HTECF-AW004). IC<sub>50</sub> values were determined by fitting dose-response curves with a  
508 four-parameter nonlinear regression model in GraphPad Prism 10.

509

#### 510 **Authentic virus neutralization assay**

511 Neutralizing activity against authentic HTNV (strain 76118) was evaluated in Vero E6  
512 cells using an immunofluorescence-based assay. Monoclonal antibodies were serially diluted  
513 2-fold in complete culture medium and incubated with HTNV at the indicated dose (100  
514 TCID<sub>50</sub> per well) for 1 h at 37 °C. The antibody and virus mixtures were subsequently trans-  
515 ferred to Vero cells seeded in 96-well plates and incubated at 37 °C for 1 h to facilitate viral  
516 entry. After incubation, remove unbound viruses by washing, replenish with fresh complete  
517 medium and incubate for another 72 hours. Following infection, culture supernatants were

518 removed, and cells were fixed and permeabilized with pre-chilled 80% acetone for 30 min at  
519 room temperature. Plates were washed twice with PBST and incubated with HTNV-NP-mAb  
520 <sup>34</sup> for 2 h at 37 °C. After washing, cells were stained with Alexa Fluor 488-conjugated goat  
521 anti-mouse IgG secondary antibody (Proteintech, Cat# SA00013-1; 1:400 dilution) for 1 h at  
522 37 °C in the dark, followed by nuclear counterstaining with DAPI (Proteintech, Cat#  
523 PR30021) for 15 min. Fluorescent signals were visualized using a fluorescence microscope.  
524 Neutralization potency was quantified by calculating IC<sub>50</sub> values using a four-parameter non-  
525 linear regression model in GraphPad Prism 10.

526

## 527 **BLI**

528 Cross-competition and binding kinetics were characterized at 30 °C using an Octet R8  
529 system (Sartorius). For epitope binning, HTNV Gn<sup>H</sup>Gc was immobilized onto HIS1K biosen-  
530 sors (Sartorius) in assay buffer (D-PBS + 0.1% BSA). Following saturation with a primary  
531 mAb, the sensors were exposed to a secondary mAb to monitor competitive binding. Data  
532 were processed using the Epitope Binning module in Octet Analysis Studio, with signal shifts  
533 recorded as relative light units (RLU).

534 For kinetic profiling, Gn<sup>H</sup>Gc -loaded sensors underwent a 120-s baseline equilibration  
535 before immersion in serial three-fold dilutions of mAbs. Association and dissociation were  
536 monitored for 300 s and 1,000 s, respectively. Sensors were regenerated with Glycine-HCl  
537 (pH 1.7) between cycles. Binding kinetics were fitted using a 1:1 Langmuir binding model in  
538 Octet Analysis Studio v13.0.2.46.

539

## 540 **Flow cytometry binding assay**

541 To evaluate antibody binding to different hantavirus glycoproteins, stable HEK293T cell  
542 lines expressing individual M-segment proteins were generated, including HTNV<sup>29</sup>, SEOV<sup>30</sup>,  
543 SNV<sup>26</sup>, ANDV<sup>27</sup>, LNV<sup>31</sup>, JUQV<sup>32</sup>, PUUV<sup>35</sup>, DOBV<sup>36</sup>, and CHOV (YP\_009506070.1).  
544 Briefly, 1 × 10<sup>6</sup> cells were collected, washed, and resuspended in 100 μL PBS. Cells were in-  
545 cubated with 10 μg primary antibodies for 1 h on ice, followed by three washes with PBS to  
546 remove unbound antibodies. Bound antibodies were detected using a PE anti-human IgG Fc  
547 Antibody (Beyotime, Cat#410708) incubated for 1 h on ice in the dark. After three additional

548 washes, cells were resuspended in PBS and analyzed on a Cytex Aurora spectral flow cytome-  
549 ter. Data were processed using FlowJo software, and antibody binding was quantified as me-  
550 dian fluorescence intensity (MFI).

551

### 552 ***In vivo* evaluation of mAb efficacy using rVSV pseudoviruses**

553 Luciferase-expressing pseudoviruses, including rVSV-HTNV, rVSV-SEOV, rVSV-SNV,  
554 and rVSV-ANDV, were generated as described above. Female BALB/c mice (6 weeks old)  
555 were inoculated intraperitoneally (i.p.) with the indicated pseudoviruses. At designated time  
556 points post-inoculation, mice were anesthetized by intraperitoneal administration of 1% pen-  
557 tobarbital sodium and subsequently injected i.p. with D-luciferin (1.5 mg per mouse; Be-  
558 yotime, Cat # ST198). Bioluminescence signals were acquired using a Lumina III in vivo im-  
559 aging system (Revvity), and three-dimensional optical tomographic reconstruction was  
560 performed using the IVIS Spectrum imaging system (PerkinElmer).

561 For antibody pre-incubation assays, pseudoviruses were incubated with monoclonal anti-  
562 bodies (6.25 mg/kg) at 37 °C for 1 h before i.p. administration into BALB/c mice. Biolumi-  
563 nescence imaging was performed 6 h post-inoculation as described above. For prophylactic  
564 protection experiments, monoclonal antibodies (6.25 mg/kg) were administered i.p. 18 h be-  
565 fore pseudovirus challenge, followed by in vivo imaging analysis 6 h after infection. The  
566 pseudoviruses challenge works were conducted in an ABSL-2 facility, where mice were pro-  
567 vided with adequate food and water throughout the experiment. Ethical approval for the ani-  
568 mal challenge study was obtained from the Biosafety Committee of Westlake University (Ap-  
569 proval ID: AP # 25-164-LDP).

570

### 571 ***In vivo* evaluation of mAb efficacy using authentic virus**

572 The therapeutic efficacy of the antibody cocktail and IgG-scFv construct was evaluated  
573 in neonatal BALB/c mice. Mice were challenged intraperitoneally with authentic HTNV  
574 (strain 76118) at a dose of  $5 \times 10^5$  PFU in 30  $\mu$ L per mouse. At 24 h post-infection, mice re-  
575 ceived intraperitoneal administration of either the antibody cocktail (6.25 mg/kg), IgG-scFv  
576 (6.25 mg/kg), or an isotype-matched human control antibody (6.25 mg/kg). Survival and body  
577 weight were monitored for 25 days. To assess viral burden and tissue distribution, brain

578 samples were collected at the endpoint for quantitative PCR analysis. All the authentic HTNV  
579 works were conducted in the ABSL-3 facility at Wuhan University, where mice were provided  
580 with adequate food and water throughout the experiment. Ethical approval for the animal  
581 challenge study was obtained from the Biosafety Committee of Wuhan University (Approval  
582 ID: AP # WQ20210048).

583

#### 584 **Immunofluorescence**

585 Tissues were fixed overnight at 4 °C in 4% paraformaldehyde, cryoprotected in 30% su-  
586 crose for 48 h, and embedded in OCT compound. Cryosections (10 µm thickness) were pre-  
587 pared for immunofluorescence analysis. Sections were fixed with 4% paraformaldehyde for  
588 10 min at room temperature, washed three times with PBS, and permeabilized with 0.25%  
589 Triton X-100. After additional PBS washes, sections were blocked with 5% rat serum at room  
590 temperature. Primary staining was performed for 1 h at room temperature using either anti-  
591 GFP antibody (Abclonal, Cat # AE012; 1:100 dilution) or ascitic fluid from HTNV-Gn-im-  
592 munized mice (1:5,000 dilution). Following three washes with PBS, sections were incubated  
593 with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (Invitrogen,  
594 Cat # A-11029; 1:2000 dilution) for 1 h at room temperature. Sections were then washed,  
595 mounted with DAPI-containing antifade mounting medium (Beyotime, Cat # P0131), and im-  
596 aged using a Zeiss LSM 900 inverted confocal microscope (Zeiss, WLL-W-20220039).

597

#### 598 **Viral RNA extraction and quantification**

599 HTNV viral RNA was quantified by quantitative polymerase chain reaction (qPCR). To-  
600 tal RNA was extracted from trizol homogenates (TaKaRa, Cat#9109) of brain tissues using  
601 the high-throughput automatic nucleic acid extraction system (Thermo Fisher Scientific,  
602 KingFisher Flex). Tissue weights were recorded before homogenization to enable normaliza-  
603 tion. Viral RNA levels were quantified by two-step RT-qPCR. Reverse transcription was per-  
604 formed using Hifair III SuperMix Plus (Yeasen, Cat# H7514240), followed by quantitative  
605 PCR using Hieff qPCR SYBR Green Master Mix (Yeasen, Cat#WH2518011). The HTNV NP  
606 segment was amplified using the following primers and probe: forward primer, 5'-CAG TGC  
607 TAC CCT GCA AAG AA-3'; reverse primer, 5'-ATT GTT CGA TAC GAT CAC TCC-3'; and

608 probe, 5'-FAM/TCA TCC TCC TTG GAT ATG CAG GCC TCA/BHQ1-3'. Reactions were  
609 carried out on an Archimed R4 Real-Time PCR Detection System (ROCGENE) in a total vol-  
610 ume of 20  $\mu$ L containing 1  $\mu$ L each of forward primer, reverse primer, and probe, 5  $\mu$ L RT-  
611 PCR enzyme mix, 5  $\mu$ L RNA template, and 7  $\mu$ L nuclease-free water. Thermal cycling condi-  
612 tions consisted of reverse transcription at 50 °C for 15 min, initial denaturation at 95 °C for 2  
613 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s, with fluorescence acquisition  
614 at each extension step. Relative viral RNA levels were determined by comparative quantifica-  
615 tion using the mean Ct values of the HTNV NP gene normalized to those of the housekeeping  
616 gene GAPDH. Relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method.

617

### 618 **Cryo-EM sample preparation and data collection**

619 For the hantavirus Gn<sup>H</sup>Gc-Fab complexes, purified Gn<sup>H</sup>Gc protein was mixed with XA-  
620 044 or XA-3H12 at a molar ratio of 1:3 and incubated at 37°C for 1 h before loading onto a  
621 Superose 200 Increase 10/300 column (GE Healthcare, 29091596) equilibrated in buffer  
622 containing 50 mM Tris and 150 mM NaCl, pH 7.4. Fractions containing the complex were  
623 concentrated using a 50-kDa molecular weight cut-off concentrator (Amicon Ultra  
624 UFC510096). Protein quality was assessed by preparative size-exclusion chromatography  
625 (Inscinstech Unique Autopre), SDS-PAGE and western blotting.

626 A 5  $\mu$ L aliquot of 0.1 mg/mL Gn<sup>H</sup>Gc-Fab complex was applied to freshly glow-  
627 discharged holy carbon grids (Quantifoil R2/2, Cu 200 mesh; 30 s at 20 mA) and plunge-  
628 frozen using a Vitrobot Mark IV (Thermo Fisher Scientific) with a blot force of 0, blot time  
629 of 2 s, 80% humidity and 4°C. Data were collected using EPU software (Thermo Fisher  
630 Scientific) to control an FEI Titan Krios G4 transmission electron microscope operated at 300  
631 kV and equipped with a Falcon 4i Summit direct electron detector and a Selectris X energy  
632 filter. Automated data collection was performed at a nominal magnification of 130,000 $\times$ ,  
633 corresponding to a pixel size of 0.97 Å. The dose rate was set to 7.78 counts per physical  
634 pixel per second. In total, 14,202 movies of the SNV Gn<sup>H</sup>Gc-XA-044 complex and 16,555  
635 movies of the HTNV Gn<sup>H</sup>Gc-XA-3H12 complex were collected, with defocus values ranging  
636 from 0.9 to 1.8  $\mu$ m.

637

## 638 **Cryo-EM model building and analysis**

639 UCSF ChimeraX and Coot were used to fit atomic models into the density maps,  
640 including XA-044 heavy chain (PDB 7S0B), XA-044 light chain (PDB 7CZU), XA-3H12  
641 heavy chain (PDB 7CZQ), XA-3H12 light chain (PDB 8R9Y), HTNV Gn (PDB 6Y6P),  
642 HTNV Gc (PDB 5LJY), SNV Gn (PDB 8AHN) and SNV Gc (PDB 6Y5W)<sup>37,38</sup>. The full  
643 complex and Gn<sup>H</sup>Gc-Fab regions were then manually rebuilt in Coot<sup>37</sup>. The models were re-  
644 fined using Rosetta<sup>39</sup>. Figures were generated using UCSF ChimeraX<sup>38</sup>. Protein-protein inter-  
645 actions have been analyzed by PISA<sup>40</sup>.

646

## 647 **Quantification and Statistical Analysis**

648 GraphPad Prism 10 software was used for data visualization and statistical analyses.  
649 Statistical comparisons between two groups were performed using unpaired, two-tailed  
650 Mann-Whitney test. Data are presented as mean ± SEM unless otherwise indicated. Statistical  
651 significance was defined as ns (not significant,  $p \geq 0.05$ ), \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p <$   
652  $0.001$ , and \*\*\*\*  $p < 0.0001$ .

653

## 654 **ACKNOWLEDGMENTS**

655 We thank the technical support from the Biomedical Research Core Facility, Laboratory  
656 Animal Resources Center, Cryo-EM Facility and High-Performance Computing Center at  
657 Westlake University. We thank Chi Biotech Co., Ltd. for assisting in the single-cell sequenc-  
658 ing. This work was supported by Westlake Education Foundation (to D.L. and Z.W.),  
659 Zhejiang Provincial Key Laboratory Construction Project (2024ZY01026), the National Natu-  
660 ral Science Foundation of China (82471858 to D.L. and 82272330 to X.W.), Zhejiang Provin-  
661 cial Natural Science Foundation of China (LR26C080001 to D.L.), the National Key R and D  
662 Program of China (2023YFC2605504 to W.H. and 2024YFA1306600 to Z.W.), the Science  
663 and Technology Program of Science, Technology and Innovation Commission of Shenzhen  
664 Municipality (JCYJ20230807090209018 to W.H.), the Shaanxi Provincial Natural Science  
665 Basic Research Program Key Project (2024JC-ZDXM-42 to R.L. and 2025JC-YBMS-988 to  
666 Z.L.), and the Assistance Program of Xijing Hospital (XJZT26JS05 to R.L.).

667

668 **DATA AVAILABILITY**

669 The data and codes that support the findings of this study are available from the corre-  
670 sponding authors on request.

671

672 **AUTHOR CONTRIBUTIONS**

673 J.Y., Z.J., Y.K., and Z.L. performed experiments. Z.D. performed computational analysis.  
674 X.Z., X.D., and J.L. collected human specimens and provided clinical support. Y.K. and Z.W.  
675 performed structural studies and analysis. Z.L., X.W., and R.L. conducted and supervised au-  
676 thentic virus *in vitro* studies. Z.J., S.C., L.N., F.L, and W.H. performed and supervised the au-  
677 thentic virus *in vivo* study. J.B., M.L., J.S., Z.Z., F.Y., R.S. and Z.K. provided critical scien-  
678 tific guidance. D.L. conceived, designed, and supervised the study. J.Y., Z.W. and D.L. wrote  
679 the paper. All authors reviewed and approved the manuscript.

680

681 **DECLARATION OF INTERESTS**

682 D.L., J.Y., R.L., Z.L., X.Z., X.W., J.L., X.D. have applied for patents concerning HTNV  
683 antibodies that are related to this work. All other authors declare no conflict of interest.

684

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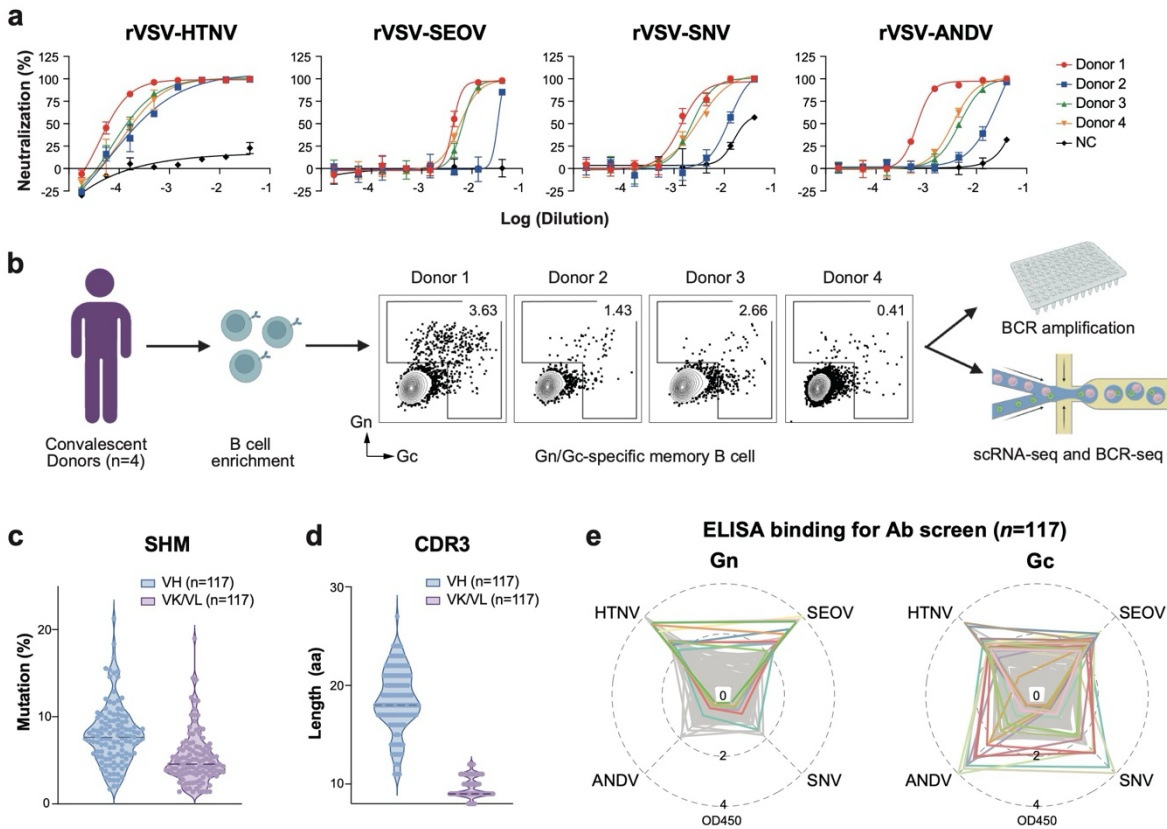
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1 **FIGURES AND FIGURE LEGENDS**



2

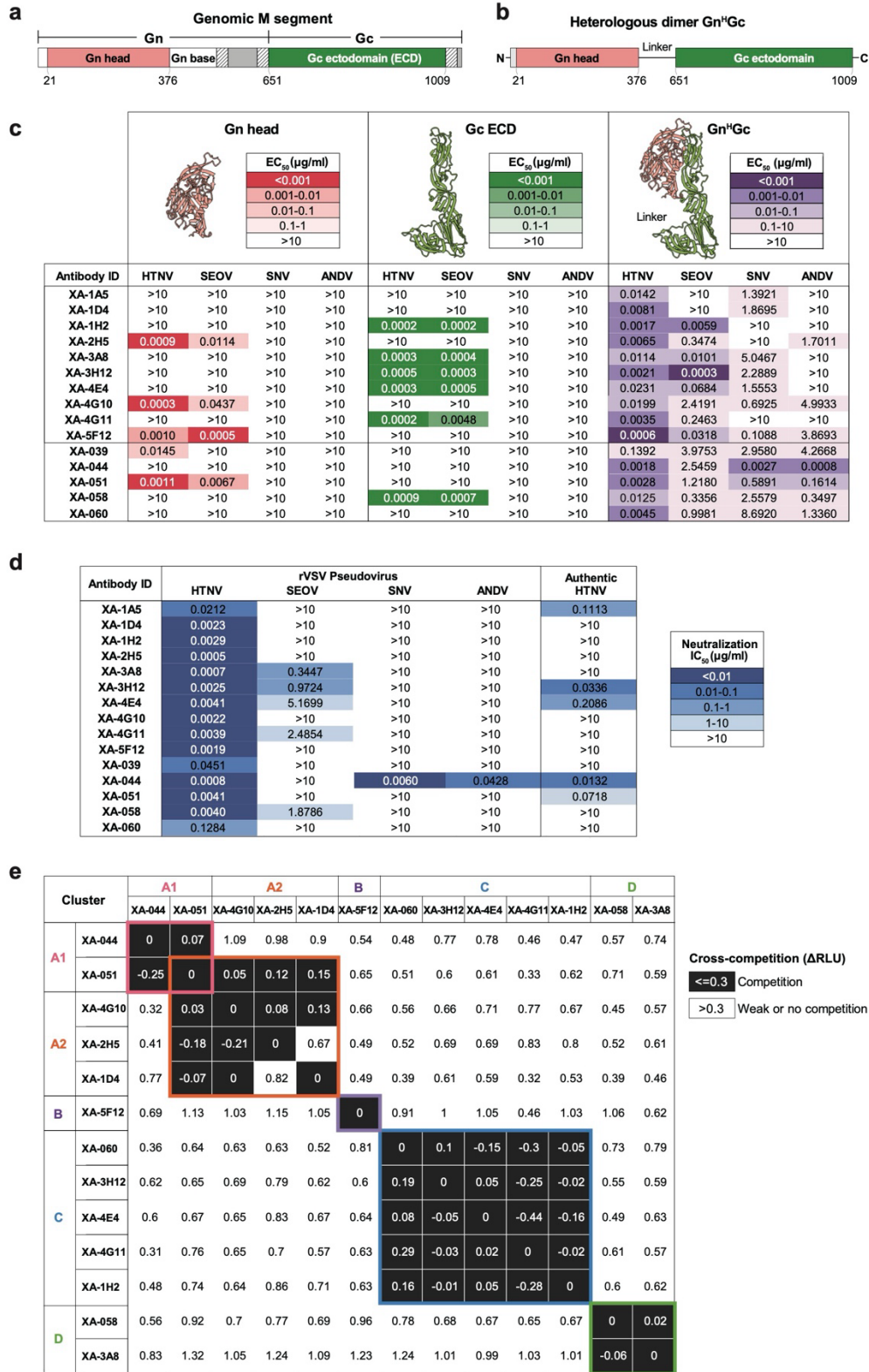
3 **Fig. 1 | Isolation of cross-reactive antibodies from HFRS convalescent patients.**

4 **a**, Plasma neutralization of GFP-encoding pseudotyped rVSV bearing HTNV, SEOV, SNV, or ANDV  
 5 glycoproteins. Plasma samples were collected from four convalescent HFRS donors and a healthy donor  
 6 as the negative control (NC). Percent neutralization was plotted against  $\log_{10}$ -transformed plasma dilution,  
 7 and relative infectivity was quantified by GFP-positive cells. Data represent mean  $\pm$  SEM from two inde-  
 8 dependent replicates.

9 **b**, Workflow for antigen-specific memory B cell isolation. B cells were enriched by negative selection,  
 10 and HTNV- or SEOV-Gn/Gc-specific IgM<sup>-</sup>IgD<sup>-</sup> B cells were sorted by FACS for single-cell PCR and  
 11 BCR sequencing.

12 **c,d**, Somatic hypermutation (SHM) frequencies (**c**) and CDR3 length distributions (**d**) in V<sub>H</sub> and V<sub>K</sub>/V<sub>L</sub>  
 13 genes of Gn/Gc-specific BCRs obtained by single-cell PCR (n=49) or single-cell BCR sequencing  
 14 (n=68).

15 **e**, ELISA-based screening of antibody binding breadth. Radar plots show antibody reactivity to Gn (left  
 16 panel) and Gc (right panel) of HTNV, SEOV, SNV, and ANDV, with concentric rings indicating OD<sub>450</sub>  
 17 values.



18

19 Fig. 2 | Identification of NABs targeting distinct hantavirus Gn/Gc neutralization sites.

20 **a**, Scheme of the hantavirus M segment domain organization, with individual protein domains indicate  
21 color-coded.

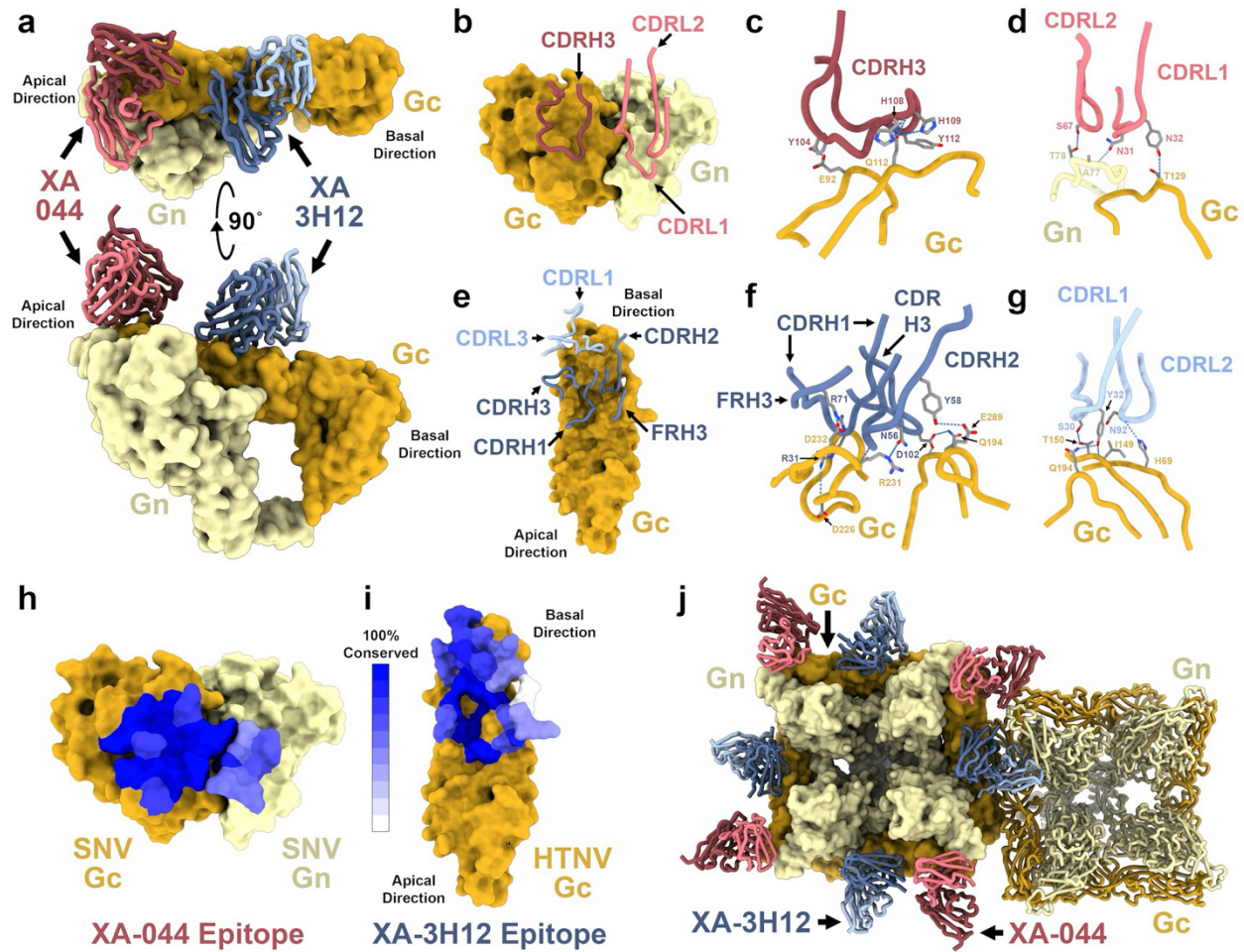
22 **b**, Scheme of the single-chain hantavirus glycoprotein heterodimer Gn<sup>H</sup>Gc construct.

23 **c**, Binding profiles of the downselected neutralizing antibodies. Recombinant Gn head, Gc ectodomain  
24 (ECD), and Gn<sup>H</sup>Gc proteins from HTNV, SEOV, SNV, and ANDV were analyzed by ELISA. Color inten-  
25 sity denotes binding EC<sub>50</sub>. Data represent mean ± SEM from two independent experiments.

26 **d**, Neutralization potency against pseudotyped rVSVs of HTNV, SEOV, SNV, and ANDV, as well as au-  
27 thentic HTNV. Color intensity denotes IC<sub>50</sub> neutralization titers. Data represent mean ± SEM from two  
28 independent experiments.

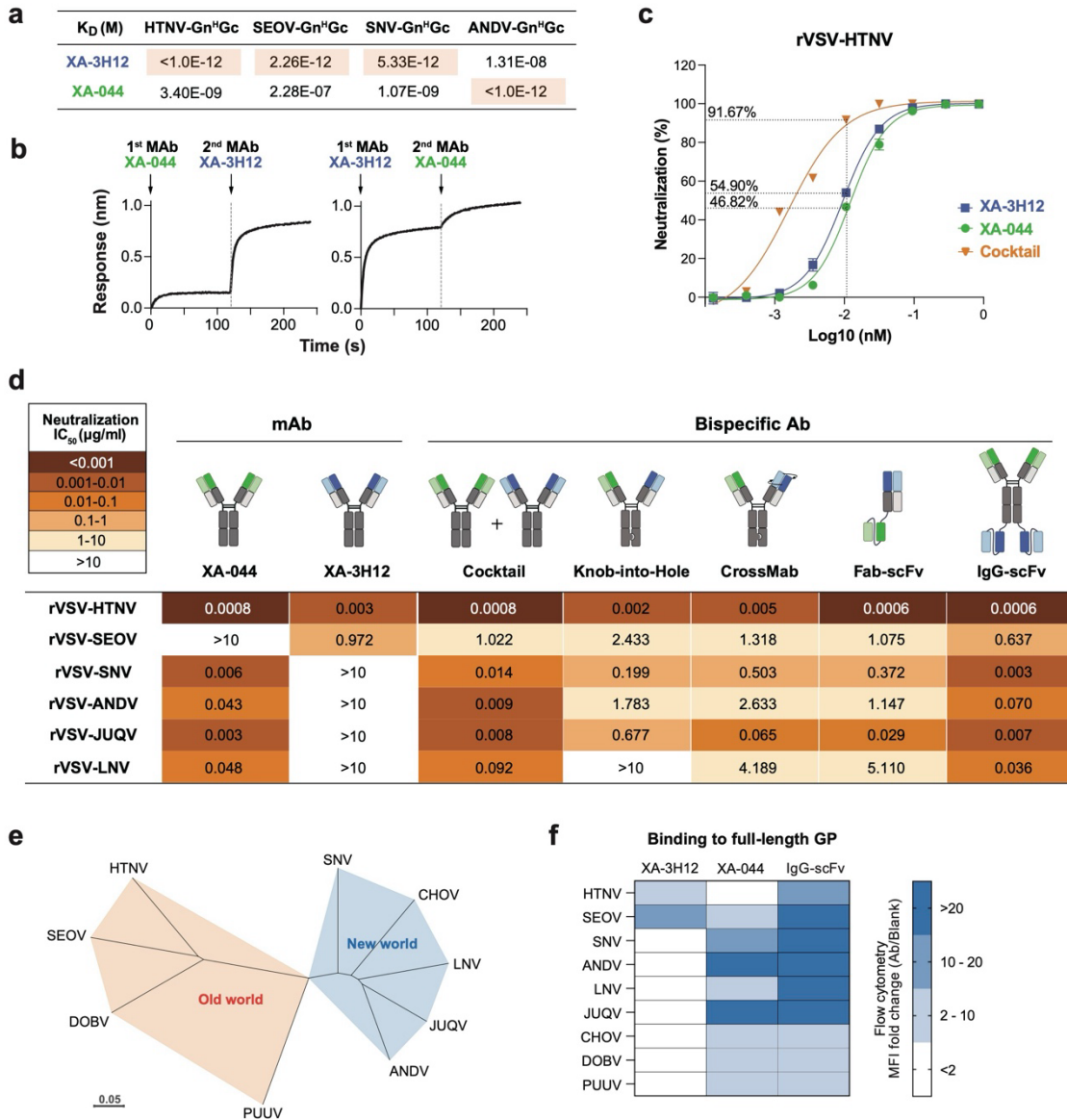
29 **e**, Epitope binning by BLI-based competition analysis. HTNV Gn<sup>H</sup>Gc antigen was captured by a primary  
30 antibody (y axis), followed by binding of a secondary antibody (x axis). Secondary-antibody responses  
31 were normalized to maximal uncompleted binding. Antibody pairs were classified as competing (black,  
32 ≤0.3) or weakly/non-competing (white, >0.3), and grouped by cross-competition profiles.

33



34  
 35 **Fig. 3 | Cryo-EM structure of the two distinct neutralization epitopes.**  
 36 **a**, XA-044 and XA-3H12 superposed onto the ANDV Gn-Gc dimer (PDB: 9P3Y), showing epitope loca-  
 37 tions. Fabs are shown as ribbons and Gn-Gc as surface; Gn, yellow; Gc, gold; XA-044 heavy/light chains,  
 38 dark/light red; XA-3H12 heavy/light chains, dark/light blue.  
 39 **b**, Focused view of XA-044 CDRH/CDRL binding at the SNV Gn-Gc apical interface.  
 40 **c,d**, Zoomed-in views of the SNV Gn-Gc interface with the XA-044 heavy (**c**) or light (**d**) chain, with se-  
 41 lected side chains shown as sticks. Hydrogen bonds and salt bridges are shown as cyan dashed lines.  
 42 **e**, Focused view of XA-3H12 CDRH/CDRL binding to the basal region of HTNV Gc domain II.  
 43 **f,g**, Zoomed-in views of the HTNV Gc interface with the XA-3H12 heavy (**f**) or light (**g**) chain, annotated  
 44 as in (**c**) and (**d**).  
 45 **h,i**, Molecular surface of XA-044 (**h**) and XA-3H12 (**i**) epitopes on SNV Gn-Gc and HTNV Gc, respec-  
 46 tively, colored by hantavirus residue conservation using a 10-step Clustal Omega gradient from deep blue  
 47 to white, corresponding to scores of 100 to 0.  
 48 **j**, XA-044 and XA-3H12 super-positioned onto ANDV Gn-Gc tetramer with neighboring tetramer (PDB

49 ID: 9P3Y and 9P3X), where main tetramer rendered in surface and neighboring tetramer in ribbon. Color  
50 scheme is the same as in (a).  
51



52

53 **Fig. 4 | Bispecific antibody strategies achieve pan-hantavirus neutralization.**

54 **a.** Binding affinities of XA-3H12 and XA-044. Binding kinetics to HTNV-, SEOV-, SNV-, and ANDV-  
55 Gn<sup>H</sup>Gc were measured by BLI and fit to a 1:1 Langmuir model. Affinities are reported as  $K_D$  values.

56 **b.** Competition analysis between XA-044 and XA-3H12 by BLI. Recombinant HTNV Gn<sup>H</sup>Gc was satu-  
57 rated with the first antibody, followed by incubation with the second neutralizing antibody to assess com-  
58 petitive binding.

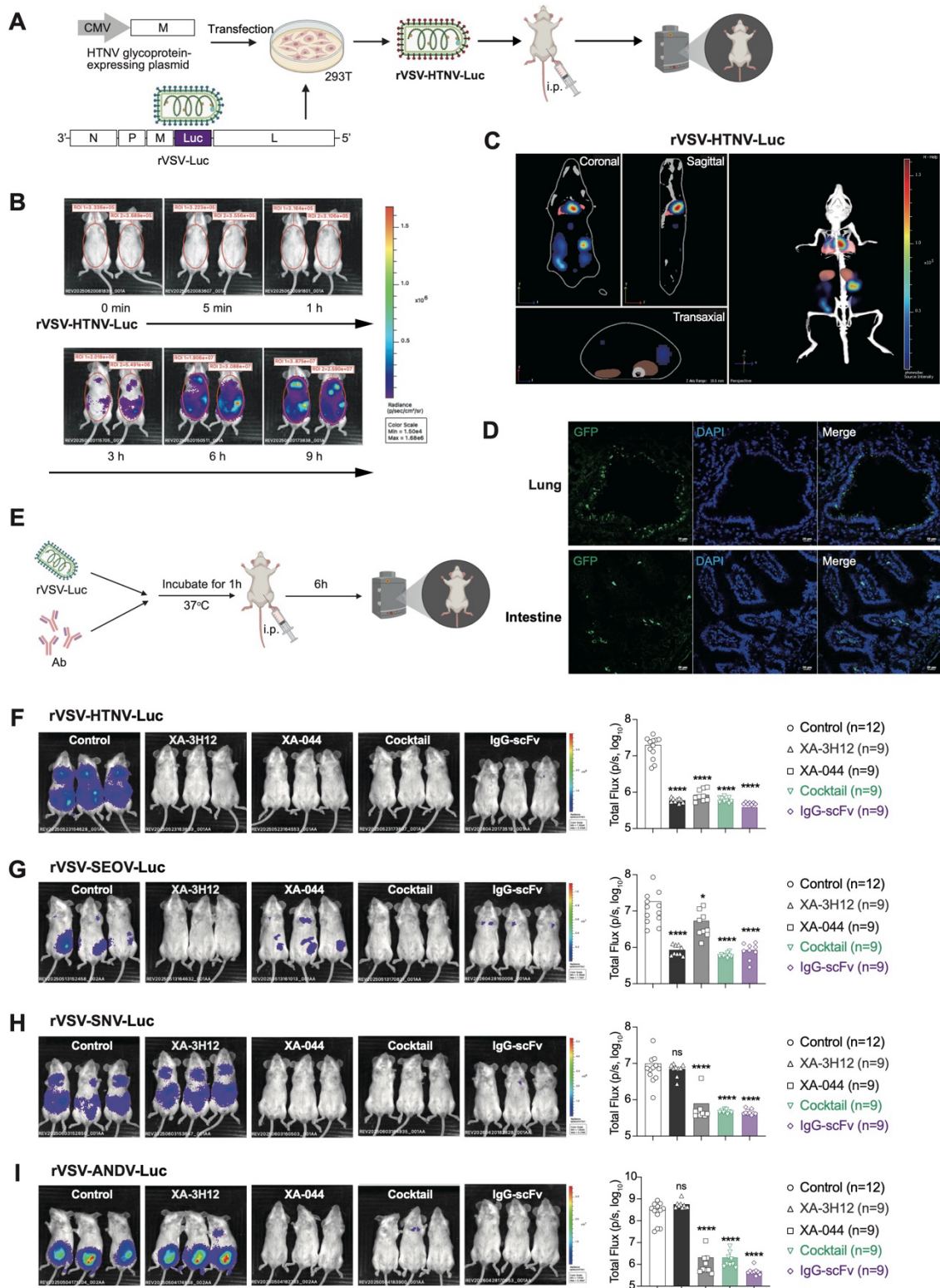
59 **c.** Synergistic neutralization by XA-044 and XA-3H12. Based on experimentally determined IC<sub>50</sub> values,  
60 XA-044 (0.0051 nM) and XA-3H12 (0.0160 nM) were mixed at a 1:1 volume ratio, using their respective  
61 IC<sub>50</sub> concentrations as the reference concentration (0.0106 nM). The mixture was then subjected to 3-fold  
62 serial dilutions above and below 1× and tested in standard neutralization assays. Neutralization (%) at the

63 combined IC<sub>50</sub> concentration is indicated by dashed lines and annotated. Data represent mean ± SEM  
64 from two independent experiments.

65 **d.** Neutralization breadth and potency of parental antibodies, antibody cocktail, and bispecific antibodies.  
66 Neutralizing activities were evaluated against six hantavirus pseudoviruses. Color intensity denotes neu-  
67 tralization IC<sub>50</sub> values across viral strains and antibody formats. Data represent mean ± SEM from two  
68 independent experiments.

69 **e,f.** Flow cytometric analysis of antibody binding to cell-surface-expressed full-length hantavirus glyco-  
70 protein (GP). **(e)** Phylogenetic analysis of representative Old World and New World hantaviruses included  
71 in the flow cytometry assay. **(f)** Heatmap summarizing antibody-binding activity, quantified by mean flu-  
72 orescence intensity (MFI) from flow cytometry.

73



74

75 **Fig. 5 | A pseudovirus reporter system enables *in vivo* evaluation of hantavirus entry blockade.**

76 **a**, Schematic of the pseudovirus *in vivo* infection model. Hantavirus glycoproteins were expressed on

77 HEK293T cells, which were then infected with luciferase-expressing rVSV-Luc to generate rVSV-

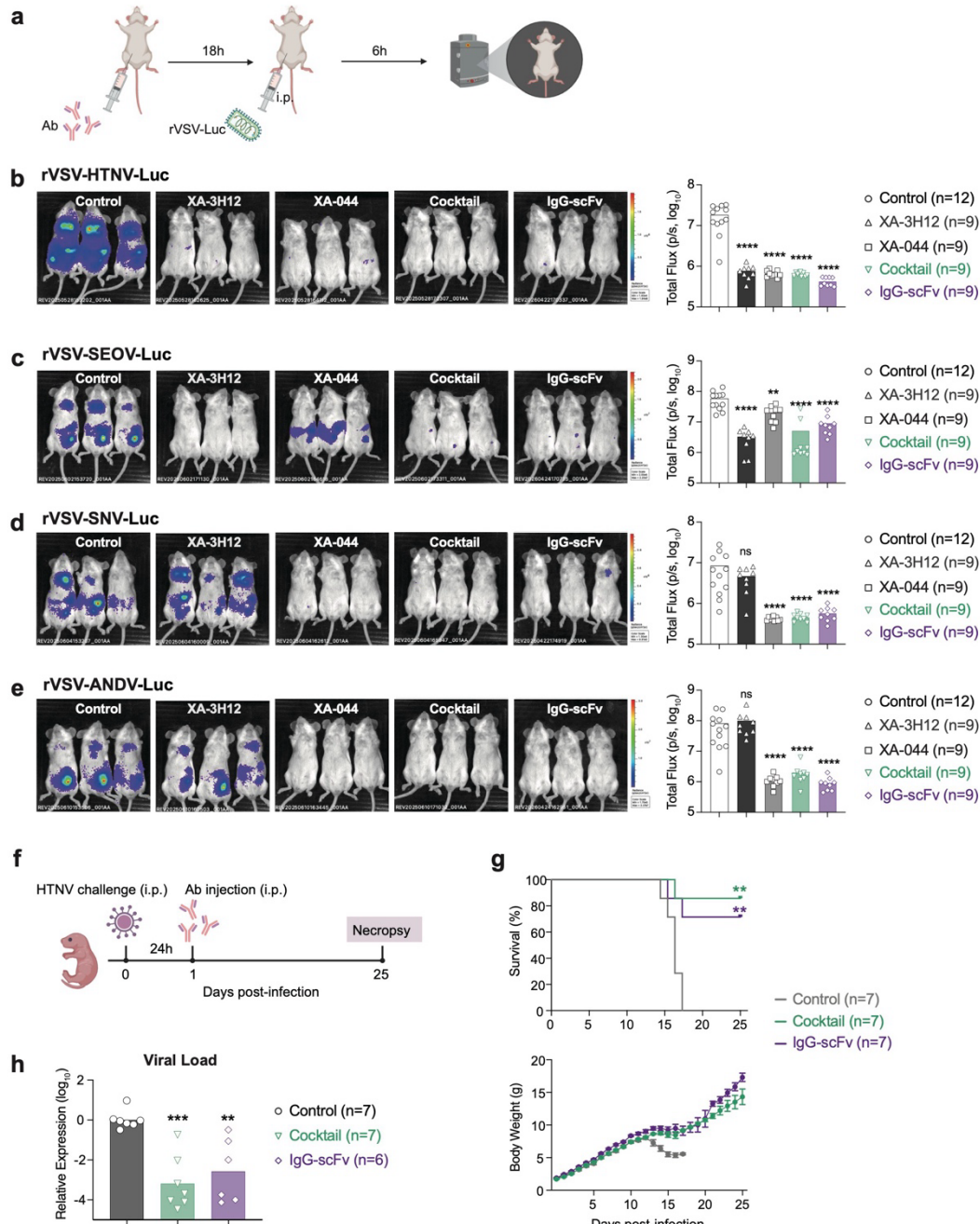
78 HTNV-Luc pseudoviruses. Pseudoviruses were administered to mice by intraperitoneal (i.p.) injection,  
79 and infection was quantified by *in vivo* bioluminescence imaging.

80 **b**, Temporal dynamics of bioluminescence in rVSV-HTNV-inoculated mice. Live-animal imaging was  
81 performed at the indicated time points, with all images displayed on the same scale.

82 **c**, Representative three-dimensional bioluminescence imaging of rVSV-HTNV-inoculated mice at 6 h  
83 post-inoculation using the IVIS Spectrum system.

84 **d**, Immunofluorescence analysis of rVSV-HTNV infection in the lung (upper panels) and intestine (lower  
85 panels) collected 6 h after inoculation. GFP-expressing rVSV-Luc pseudoviruses were used. GFP-positive  
86 signals are shown in green, and nuclei were counterstained with DAPI (blue). Scale bars, 75  $\mu$ m.

87 **e-i** Antibody-mediated blockade of pseudovirus entry in mice. **(e)** Experimental design. Luciferase-ex-  
88 pressing **(f)** rVSV-HTNV-Luc, **(g)** rVSV-SEOV-Luc, **(h)** rVSV-SNV-Luc, or **(i)** rVSV-ANDV-Luc pseu-  
89 doviruses were preincubated with neutralizing antibodies before i.p. administration into BALB/c mice.  
90 Bioluminescence was measured at 6 h post-inoculation. Representative images are shown on the left, with  
91 quantified bioluminescence intensities on the right. Statistical significance was determined using the  
92 Mann-Whitney test. ns, not significant;  $p \geq 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .  
93

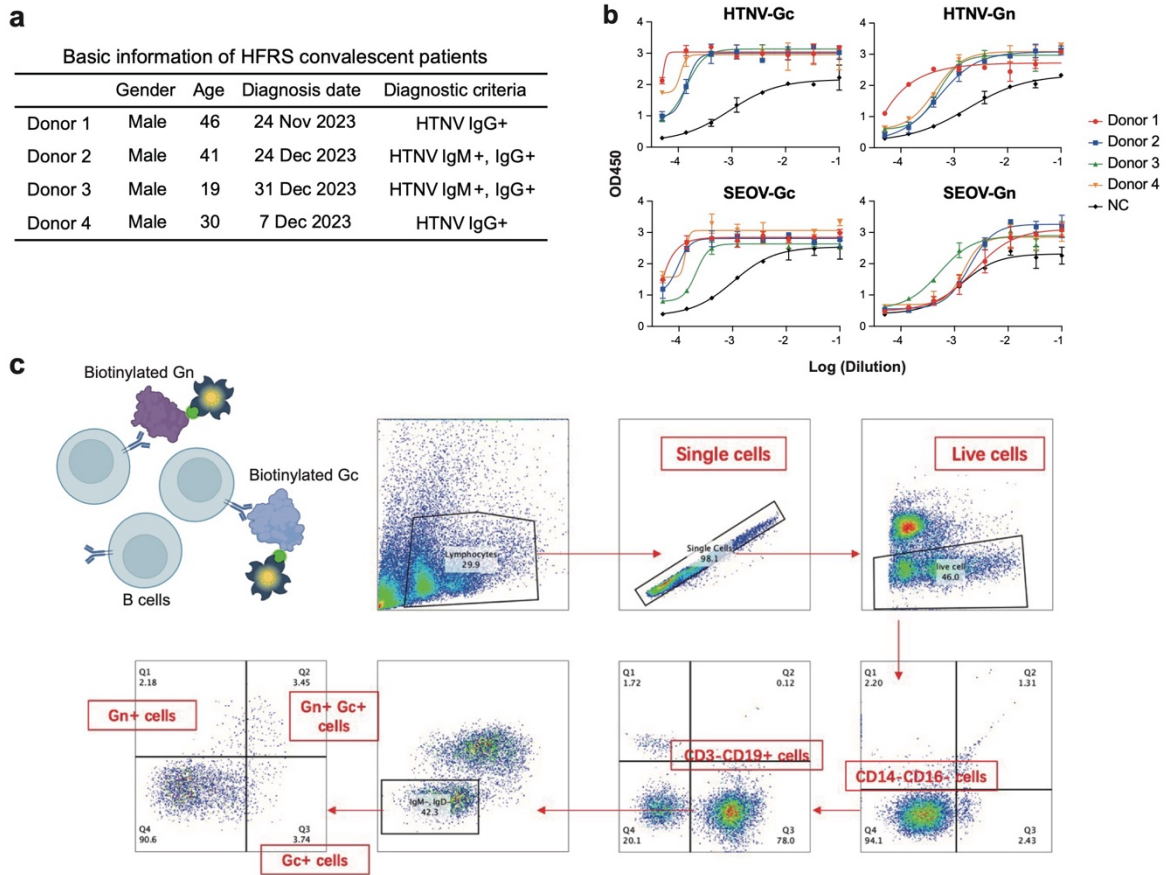


94

95 **Fig. 6 | Bispecific strategies confer broad protection *in vivo*.**

96 **a-e**, Prophylactic treatment prevented pseudovirus entry in mice. **(a)** Experimental design. Antibodies  
 97 were i.p. administered into BALB/c mice 18 h prior to challenge with luciferase-expressing **(b)** rVSV-  
 98 HTNV-Luc, **(c)** rVSV-SEOV-Luc, **(d)** rVSV-SNV-Luc, or **(e)** rVSV-ANDV-Luc pseudoviruses. Bio-lumi-  
 99 nescence was measured at 6 h post-inoculation. Representative images are shown on the left, with quanti-  
 100 fied bioluminescence intensities on the right.

101 **f-i**, Post-exposure therapy protected neonatal mice from lethal HTNV challenge. **(f)** Experimental design.  
102 Neonatal BALB/c mice were challenged i.p. with authentic HTNV and received i.p. administration of the  
103 antibody cocktail, IgG-scFv, or isotype control antibody at 24 h post-infection. **(g)** Survival curves and  
104 body weight changes. **(h)** Viral RNA loads in brain. Statistical significance was determined using the  
105 Mann-Whitney test. ns, not significant;  $p \geq 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .  
106



108

109 **Extended Data Fig. 1 | Antigen-specific B cell sorting from HFRS convalescent donors.**

110 **a**, Clinical characteristics of four HFRS convalescent donors diagnosed in 2023. Donors were confirmed  
 111 to be HTNV IgG-positive and/or IgM-positive during hospitalization. Peripheral blood was collected dur-  
 112 ing convalescence, approximately 6 months after discharge, after informed consent.

113 **b**, Plasma binding to HTNV and SEOV Gn and Gc from four convalescent HFRS donors was measured  
 114 by ELISA. Plasma from a healthy donor was used as the negative control (NC). Data represent mean ±  
 115 SEM from two independent experiments.

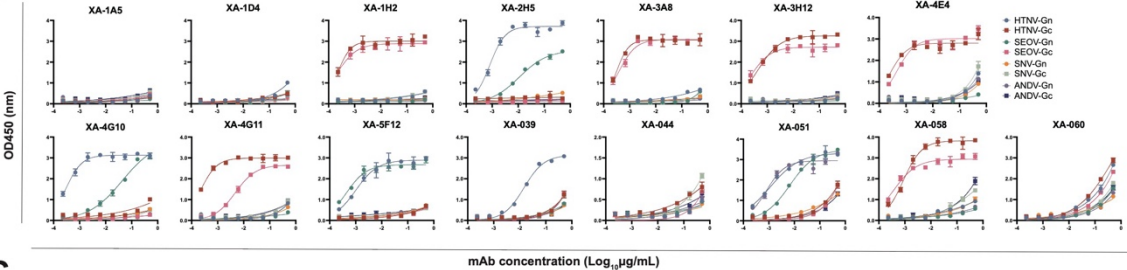
116 **c**, Representative flow-cytometric gating strategy for single-cell sorting of antigen-specific memory B  
 117 cells recognizing HTNV-Gn, HTNV-Gc, SEOV-Gn, or SEOV-Gc.

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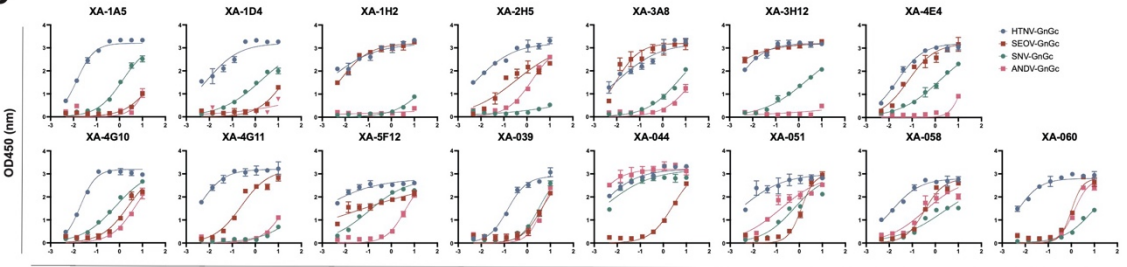
**A**

Group	MAb	Donor	Heavy chain variable gene sequence				Light chain variable gene sequence					
			CDR3 length (aa)	Mutation rate (%)	V-gene and allele	J-gene and allele	CDR3 length (aa)	Mutation rate (%)	V-gene and allele	J-gene and allele		
Single cell PCR	1H2	Donor 1			IGHV3-30*18	IGHJ3*02	QAWDRSTVI	9	93.80	IGLV3-1*01	IGLJ2*01	
	1D4	Donor 1	ARSCYGGGCTYYMYMDV	17	94.85	IGHV1-69*09	IGHJ3*03	GTWETIGRAWV	11	94.22	IGLV1-51*01	IGLJ3*02
	4G10	Donor 4	VRDSYCSGGSCYSTNWFDL	20	94.24	IGHV3-33*01	IGHJ5*02	TSYTSRSTVL	10	95.92	IGLV2-14*03	IGLJ2*01
	3A8	Donor 3	ARDQGWFAFDY	11	95.61	IGHV3-21*01	IGHJ4*02	VTWDDSLNLIL	11	95.56	IGLV1-44*01	IGLJ2*01
	2H5	Donor 2	ARDQTGSWASGF	13	91.55	IGHV3-21*01	IGHJ4*02	SSYVGTNNVI	10	96.26	IGLV2-8*01	IGLJ2*01
	1A5	Donor 1	ARSSGWFGAFDI	14	93.17	IGHV1-3*01	IGHJ3*02	QQSYSVPT	8	94.37	IGKV1-39*01	IGKJ1*01
	3H12	Donor 3	ASTPGYDTPWYGPVR	15	93.22	IGHV3-44D*06	IGHJ4*02	QKYNMAPFT	9	97.89	IGKV1-27*01	IGKJ3*01
	4E4	Donor 4	SAKDSQYSSQVRDVFVDV	18	90.97	IGHV3-53*04	IGHJ3*01	QKYNAPWIT	9	97.89	IGKV1-27*01	IGKJ1*01
	4G11	Donor 4	VCDYMWGTFLDY	12	89.09	IGHV3-53*04	IGHJ4*02	QNYDHWPKT	9	93.71	IGKV1-27*01	IGKJ1*01
	5F12	Donor 4	ARATFNQYVWQYKRDYLDY	21	98.30	IGHV3-33*01	IGHJ4*02	QQYNSYSRT	9	95.80	IGKV1-5*04	IGKJ1*01
Single cell BCR-seq	HT-SNY-38	/	AREAVASRTYNNWFDP	17	85.03	IGHV4-59*01	IGHJ5*02	QSYDSSLGSGV	11	95.65	IGLV1-40*01	IGLJ3*02
	HT-SNY-44	/	ATGVRYGELHPQYFYGMVDV	21	91.78	IGHV3-23*01, IGHV3-23*02, IGHV3-23D*01	IGHJ3*02	QQSFSPWIT	9	94.70	IGKV1-39*01, IGHV1D-39*01	IGKJ1*01
	HT-SNY-51	/	TGYSGASCYSPYLYLDN	18	91.22	IGHV3-30*18, IGHV3-30*5*01	IGHJ4*02	QQSYSPWIT	9	94.72	IGKV1-39*01, IGHV1D-39*01	IGKJ1*01
	HT-SNY-58	/	ARDRGGQRNDNWSRFYMDV	19	91.64	IGHV4-61*02	IGHJ3*03	QQSFQTPYT	9	95.78	IGKV1-39*01, IGHV1D-39*01	IGKJ2*01
	HT-SNY-60	/	VRFGCSRSTQCRPNYFDP	19	91.53	IGHV3-30*03, IGHV3-30*04, IGHV3-30*19	IGHJ5*02	GSYTGSTWV	10	95.92	IGLV2-14*03	IGLJ3*02

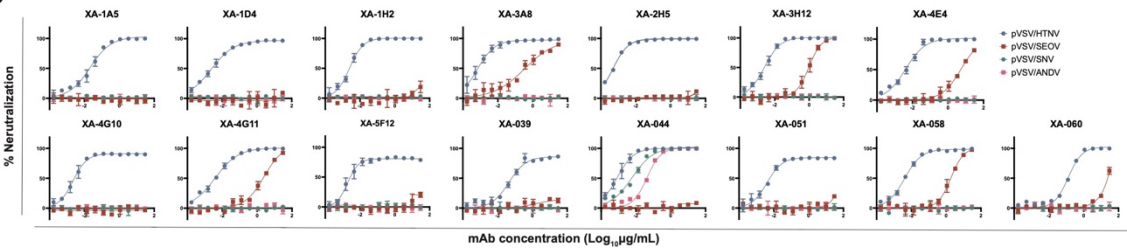
**B**



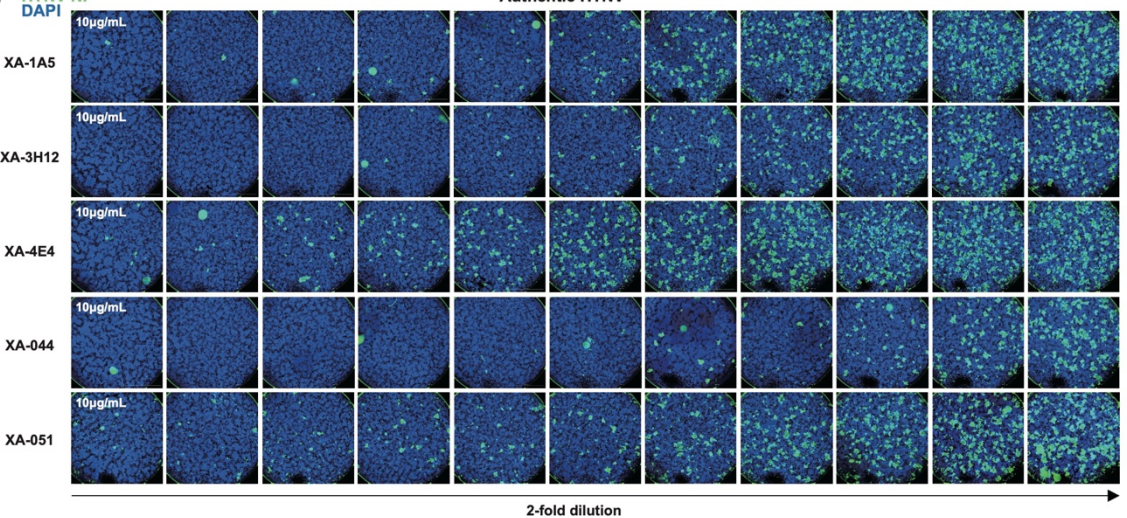
**C**



**D**



**E**



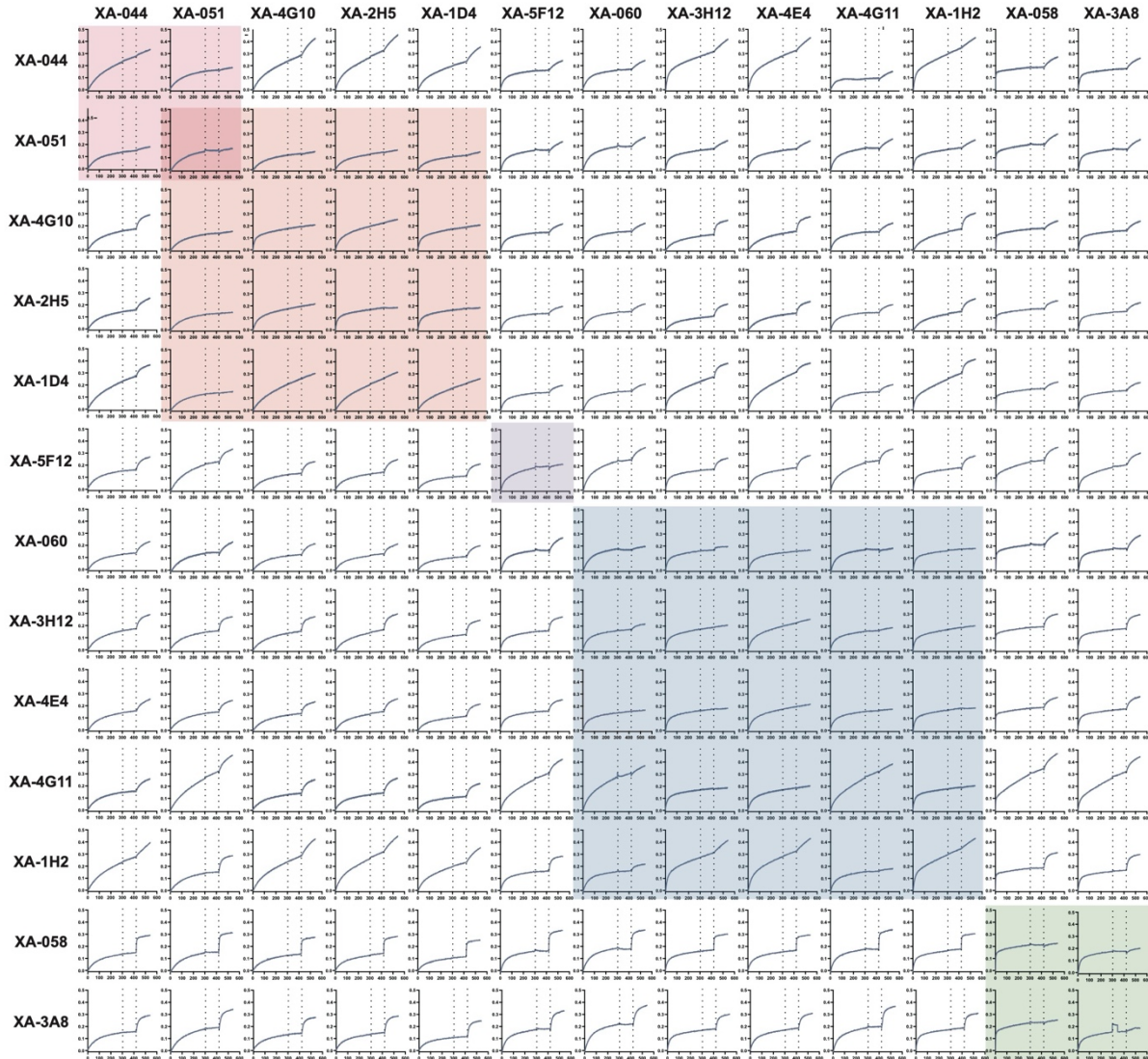
120 **Extended Data Fig. 2 | Characterization of isolated neutralizing antibodies.**

121 **a**, Immunogenetic features of 15 antigen-specific BCR sequences isolated from HFERS convalescent do-  
122 nors, including donor origin, heavy- and light-chain CDR3 amino acid sequences and lengths, somatic  
123 hypermutation frequencies, and V/J-gene usage and alleles.

124 **b,c**, Representative ELISA binding curves of isolated mAbs against recombinant Gn and Gc (**b**) or Gn<sup>H</sup>Gc  
125 proteins (**c**). Data are mean  $\pm$  SEM from two independent experiments; corresponding EC<sub>50</sub> values are  
126 summarized in Fig. 2c.

127 **d**, Representative neutralization curves of isolated mAbs against rVSV-HTNV-GFP, rVSV-SEOV-GFP,  
128 rVSV-SNV-GFP, and rVSV-ANDV-GFP pseudoviruses. Data are mean  $\pm$  SEM from two independent ex-  
129 periments; corresponding IC<sub>50</sub> values are summarized in Fig. 2d.

130 **e**, Representative immunofluorescence images showing neutralization of authentic HTNV. Vero E6 cells  
131 were infected with HTNV (strain 76118) preincubated with neutralizing antibodies, and infection was  
132 quantified by immunofluorescent staining of HTNV Nucleocapsid protein (NP).



133

134

**Extended Data Fig. 3 | BLI-based epitope competition analysis of neutralizing antibodies.** Recombi-

135

nant HTNV Gn<sup>H</sup>Gc protein was immobilized via binding to a primary neutralizing antibodies, followed

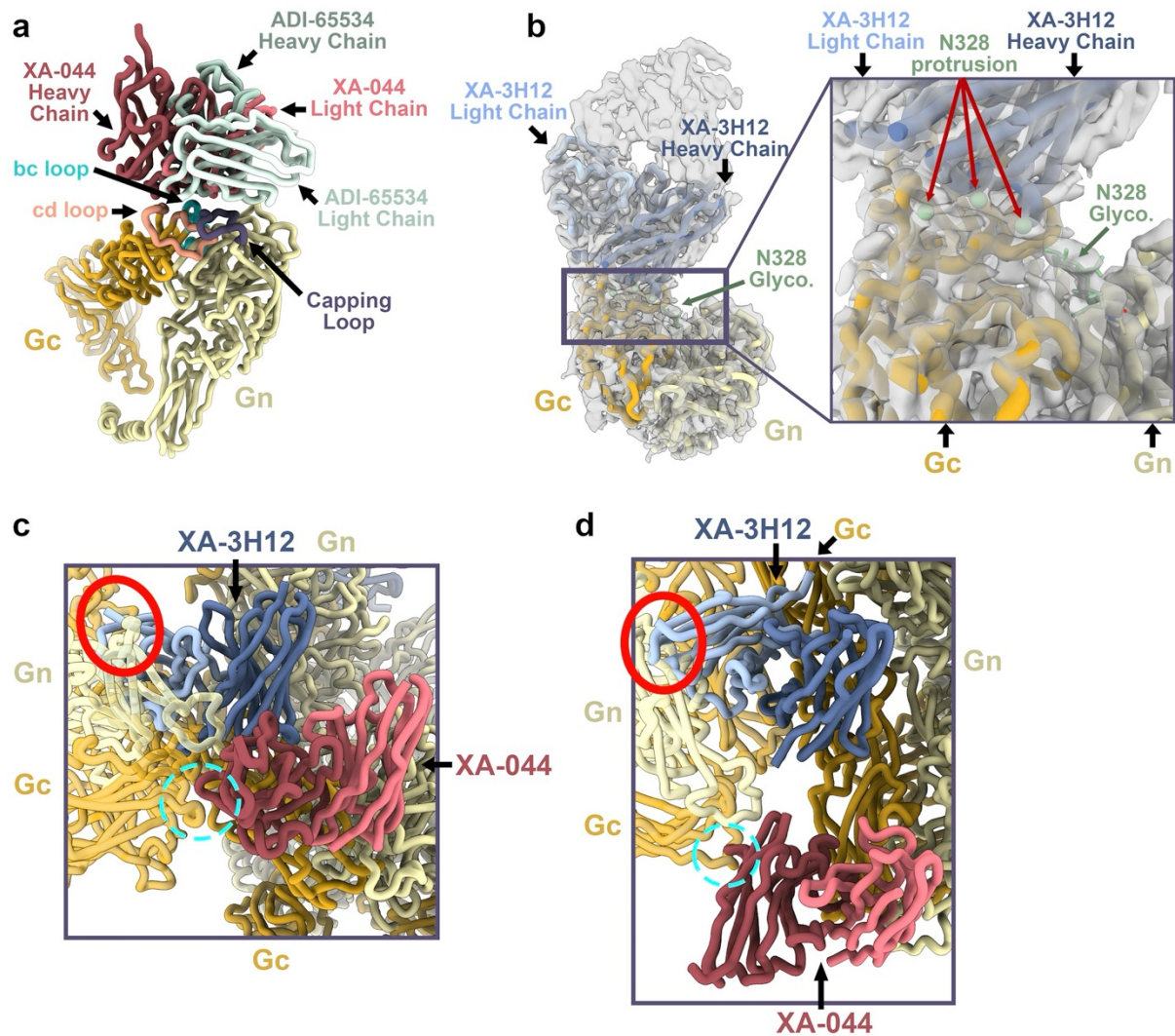
136

by binding assessment of a second neutralizing antibodies. Epitope competition was quantified as the dif-

137

ference in response ( $\Delta$ RLU) between sequential antibody-binding steps. Related to Fig. 2e.

138



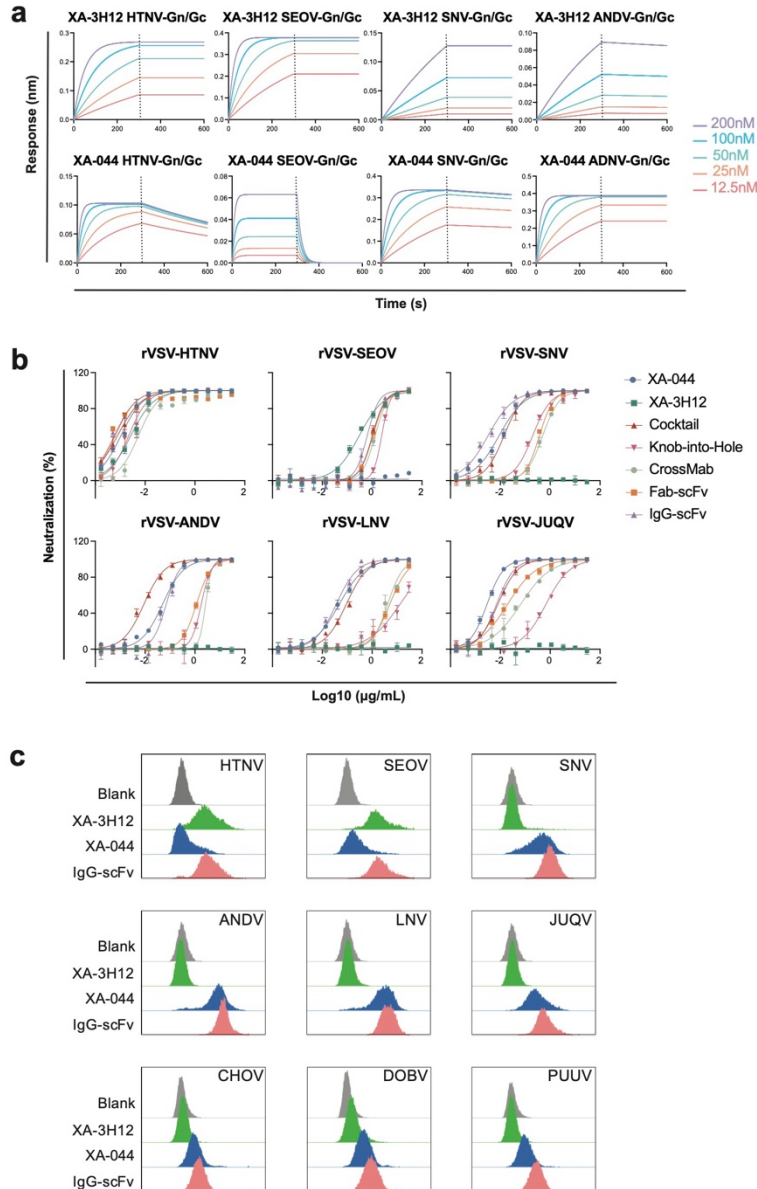
139

140 **Extended Data Fig. 4 | Focused analysis of XA-044 and XA-3H12 epitopes and neutralization mech-**  
 141 **anism.**

142 **a**, Superposition of XA-044 on the ANDV Gn-Gc dimer bound by ADI-65534, showing overlapping  
 143 epitopes with XA-044 shifted slightly toward the Gc fusion loops (bc and cd loops). Gn: yellow, Gc:  
 144 gold; XA-044 heavy and light chains: dark and light red; ADI-65534 heavy and light chains: dark and  
 145 light mint; Gc bc loop: cyan, Gc cd loop: salmon.

146 **b**, The N-glycan protruding from N328 of HTNV Gn interacts with the XA-044 heavy chain. Left, overall  
 147 view; right, zoomed-in view with the XA-3H12 Fab-HTNV Gn<sup>H</sup>Gc complex fitted into the cryo-EM  
 148 density map. The purple rectangle indicates the zoomed-in region. Built glycans are shown as green  
 149 sticks, and hypothesized glycan protrusions are shown as green dots.

150 **c,d**, Zoomed-in views of steric clashes between XA-044 or XA-3H12 and a neighboring Gn-Gc tetramer  
151 in the lattice, with XA-044 in red, XA-3H12 in blue, Gn in yellow, and Gc in gold. Cyan circles indicate a  
152 potential minor clash between XA-044 and the neighboring tetramer, whereas red circles indicate major  
153 clashes between XA-3H12 and the neighboring tetramer.  
154



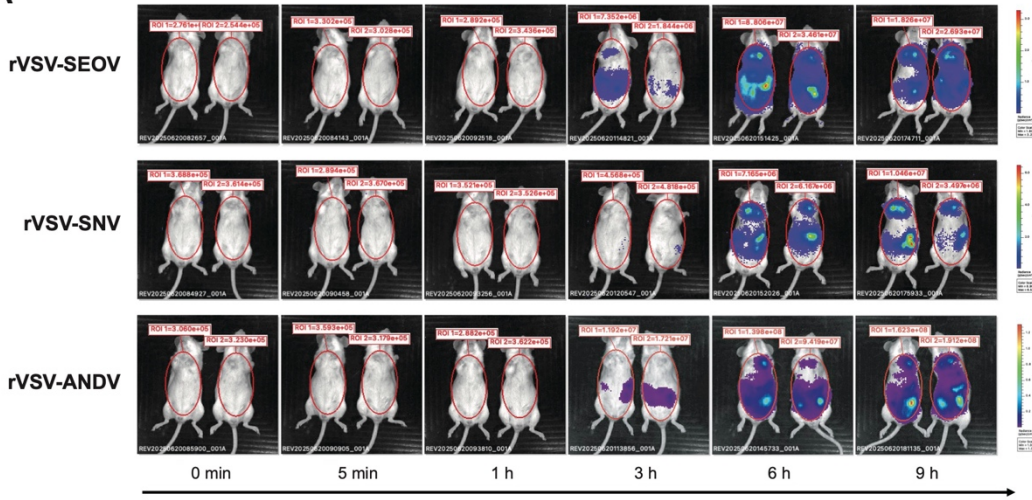
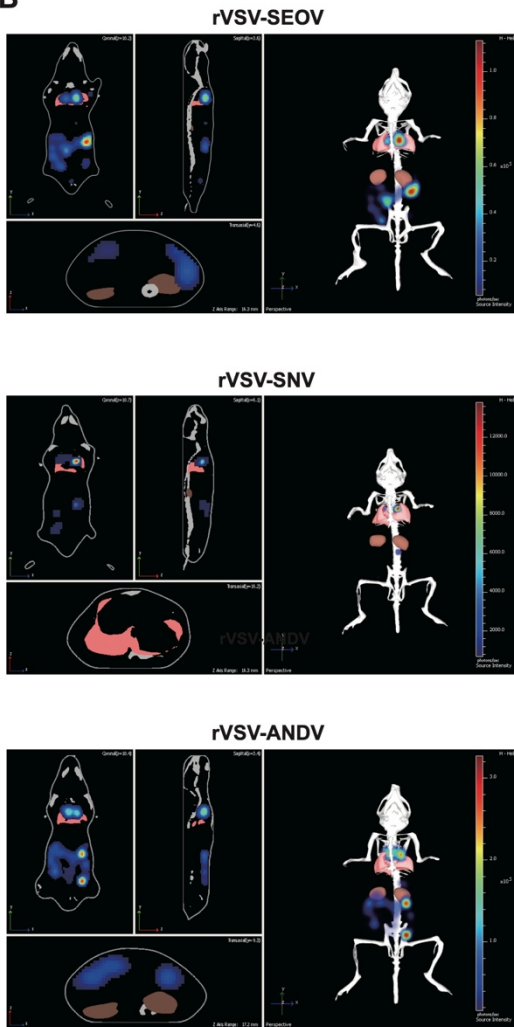
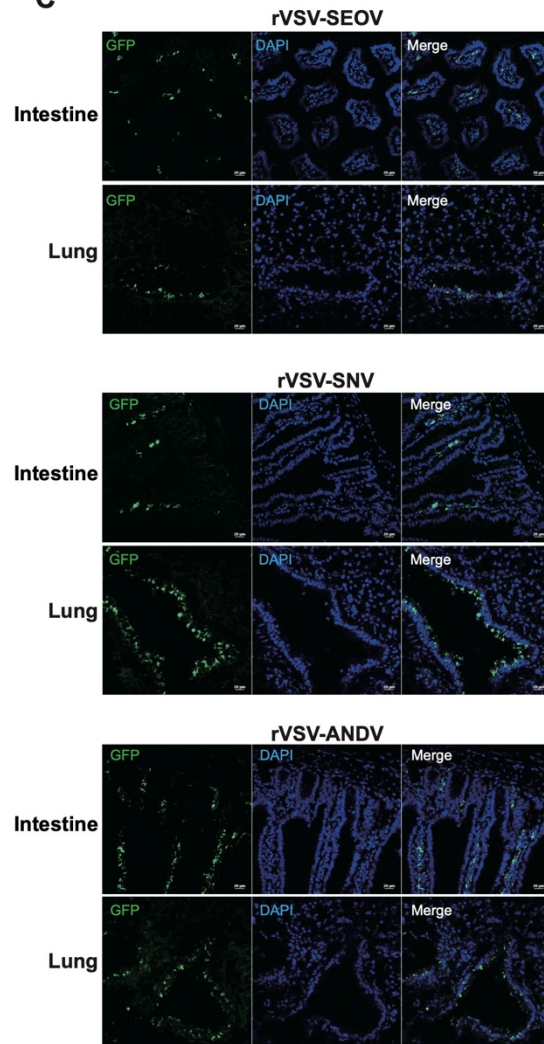
155

156 **Extended Data Fig. 5 | Characterization of selected NAb and bispecific antibodies.**

157 **a**, BLI analysis of NAb binding to HTNV-, SEOV-, SNV-, and ANDV-Gn<sup>H</sup>Gc. Binding kinetics were  
 158 measured using 2-fold serial dilutions of recombinant Gn<sup>H</sup>Gc antigens (200-12.5 nM) and fit to a 1:1  
 159 Langmuir model. Binding affinities are reported as  $K_D$  values.

160 **b**, Representative neutralization curves of parental NAbs, an antibody cocktail, and bispecific antibodies  
 161 against hantavirus pseudoviruses, including rVSV-HTNV, rVSV-SEOV, rVSV-SNV, rVSV-ANDV, rVSV-  
 162 JUQV, and rVSV-LNV. Data are mean  $\pm$  SEM from two independent experiments; corresponding  $IC_{50}$   
 163 values are summarized in Fig. 3d.

164 **c**, Flow cytometric analysis of antibody binding to full-length hantavirus glycoprotein (GP). HEK293T  
165 cells expressing the indicated GP were incubated with individual antibodies followed by fluorophore-con-  
166 jugated secondary antibody staining. Colored histograms indicate specific binding, and the gray histogram  
167 indicates the negative control. Related to Figure 3f.  
168

**A****B****C**

170 **Extended Data Fig. 6 | Establishment of a pseudovirus-based *in vivo* infection system.**  
171 **a,** Temporal dynamics of bioluminescence signals in rVSV-SEOV-Luc, rVSV-SNV-Luc, and rVSV-  
172 ANDV-Luc infected mice. *In vivo* bioluminescence imaging was performed at the indicated time points  
173 after inoculation. All images were processed and displayed using the same intensity scale.  
174 **b,** Representative 3D images of mice inoculated with rVSV-SEOV-Luc, rVSV-SNV-Luc, or rVSV-  
175 ANDV-Luc at 6 h post-inoculation.  
176 **c,** Immunofluorescence analysis of rVSV-SEOV-GFP, rVSV-SNV-GFP, and rVSV-ANDV-GFP infec-  
177 tion in the intestine (upper panels) and lung (lower panels) collected 6 h after inoculation. GFP-positive  
178 signals are shown in green, and nuclei were counterstained with DAPI (blue). Scale bars, 75  $\mu$ m.  
179

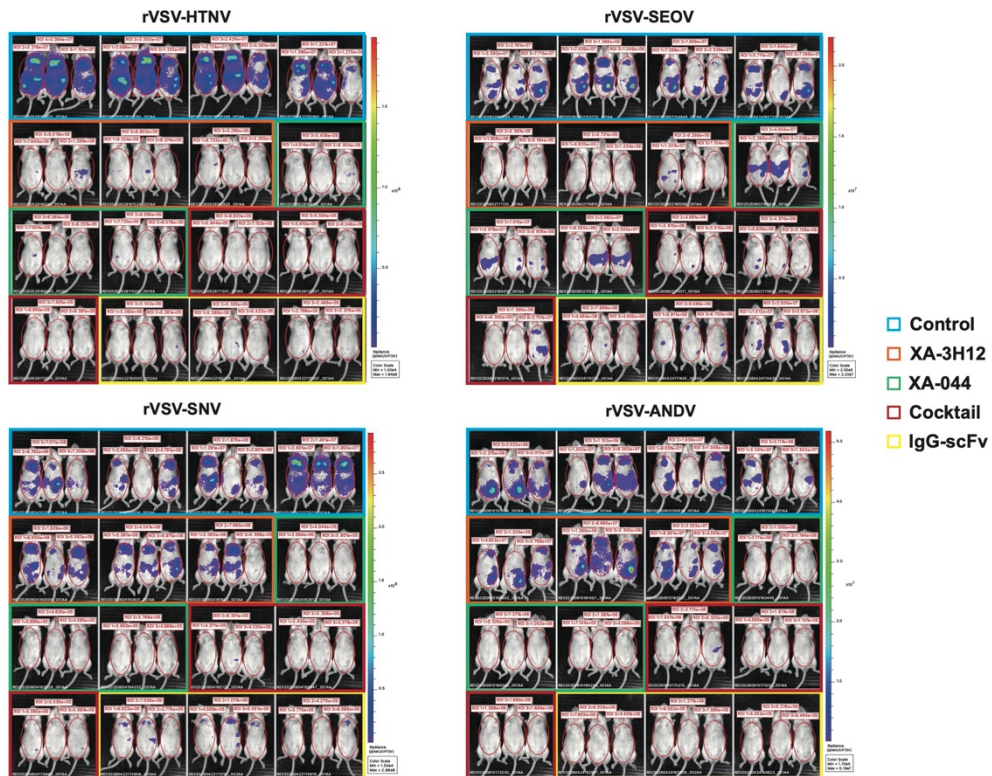
**a**

**With incubation**



**b**

**Prophylaxis**



181 **Extended Data Fig. 7 | Bioluminescence imaging related to Figures 4 and 5.**

182 **a**, Complete dataset for antibody-mediated blockade of pseudovirus entry shown in Fig. 4f-i.

183 **d**, Complete dataset for the prophylactic antibody protection study shown in Fig. 5b-e.

184

185

186 **Extended Data Table 1 | Immunogenetic analysis and experimental data of the hantavirus Gn/Gc-**  
187 **specific antibodies.**

188

189 **Extended Data Table 2 | Cryo-EM data collection, processing and model refinement statistics.**

190