

1 A novel experimental system reveals immunoregulatory responses as mediators of
2 persistent orthohantavirus infections in a rodent reservoir host

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4 Tomas Strandin¹, Teemu Smura¹, Paula Ahola¹, Kirsi Aaltonen^{1,2}, Tarja Sironen^{1,2}, Jussi
5 Hepojoki^{1,3}, Isabella Eckerle⁴, Rainer G. Ulrich⁵, Olli Vapalahti^{1,2}, Anja Kipar^{2,3}, Kristian M.
6 Forbes⁶

7

8 1) Zoonosis Unit, Department of Virology, Medicum, University of Helsinki, Helsinki, Finland

9 2) Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, University of
10 Helsinki, Helsinki, Finland

11 3) Laboratory for Animal Model Pathology, Institute of Veterinary Pathology, Vetsuisse
12 Faculty, University of Zurich, Zurich, Switzerland

13 4) Institute of Virology, University of Bonn Medical Centre, Bonn, Germany and present
14 address: Geneva Centre for Emerging Viral Diseases, University Hospital of Geneva &
15 Department of Microbiology and Molecular Medicine, University of Geneva, Geneva,
16 Switzerland

17 5) Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Federal
18 Research Institute for Animal Health, Greifswald-Insel Riems, Germany

19 6) Department of Biological Sciences, University of Arkansas, Fayetteville, USA

20 Word count: abstract 250, text 6019

21 Corresponding author

22 Tomas Strandin

23 tomas.strandin@helsinki.fi

24 Running title: Persistence of Puumala orthohantavirus in bank voles

25 Keywords: immunity, Puumala orthohantavirus, rodent reservoir, spillover, vole, zoonoses

26 Abstract

27 Orthohantaviruses are globally emerging zoonotic pathogens. Human infections are characterized
28 by an overt immune response that is efficient at counteracting virus replication but can also cause
29 severe tissue damage. In contrast, orthohantavirus infections in rodent reservoir hosts are persistent
30 and asymptomatic. The mechanisms facilitating asymptomatic virus persistence in reservoir hosts
31 are not well understood but could help to guide therapeutic strategies for human infections. Here we
32 report on a study using *in vivo* and *in vitro* experiments to investigate immune responses associated
33 with persistent Puumala orthohantavirus (PUUV) infections in the bank vole (*Myodes glareolus*), its
34 reservoir host. We examined adaptive cellular and humoral responses by quantifying changes in T-
35 cell related gene expression in the spleen and immunoglobulin (Ig) responses in blood, respectively.
36 Since existing Vero E6-cell adapted hantavirus isolates have been demonstrated to have lost their
37 wild-type infection characteristics, infections were conducted with a novel PUUV strain isolated on
38 a bank vole cell line. Whole virus genome sequencing revealed that only minor sequence changes
39 occurred during the isolation process, and critically, experimental infections of bank voles with the
40 new isolate resembled natural infections. *In vitro* infection of bank vole splenocytes with the novel
41 isolate demonstrated that PUUV promotes immunoregulatory responses by inducing interleukin-10,
42 a cytokine strongly associated with chronic viral infections. A delayed virus-specific humoral
43 response occurred in experimentally infected bank voles, which is likely to allow for initial virus
44 replication and the establishment of persistent infections. These results suggest that host
45 immunoregulation facilitates persistent orthohantavirus infections in reservoir hosts.

46

47 Importance

48 Orthohantaviruses are a group of global pathogens that regularly spillover from rodent reservoirs
49 into humans and can cause severe disease. Conversely, infections in reservoir hosts do not cause
50 obvious adverse effects. The mechanisms responsible for persistent asymptomatic reservoir

51 infections are unknown, and progress has been hindered by the absence of an adequate experimental
52 system. Knowledge on these mechanisms could help provide strategies to treat human infections.
53 We developed and validated an experimental system based on an orthohantavirus isolated in cells of
54 its vole reservoir host. Using animal and cell culture experiments in the reservoir host system, we
55 demonstrated that infection suppresses immunity in the vole reservoir via specific mechanisms,
56 likely allowing the virus to take hold and preventing immune responses that can cause self-damage.

57

58 Introduction

59 Understanding how zoonotic pathogens are maintained and transmitted in nature is critical for
60 efforts to curtail human disease (1). Most investigations have focused on the identification of
61 wildlife reservoir hosts (2, 3), and to a lesser extent, the characterization of high-risk subgroups and
62 time periods, such as migration and breeding periods (4, 5), when transmission is greatest in
63 reservoir host populations and the risk of human exposure may therefore be elevated. However, it
64 remains largely unknown how zoonotic pathogens are maintained at an individual-level in wildlife
65 reservoirs, such as the molecular and cellular mechanisms of host-pathogen interactions that
66 promote clearance or persistence, and feed back onto these population-level dynamics.

67

68 Hantaviruses (genus *Orthohantavirus*, order *Bunyavirales*) provide one of the most clearly
69 described reservoir host-zoonotic pathogen relationships (6). Hantavirus species have been
70 identified across the globe, some of which cause Hemorrhagic Fever with Renal Syndrome (HFRS)
71 in Eurasia or Hantavirus Cardiopulmonary Syndrome (HCPS) in the Americas (7). These diseases
72 begin abruptly with a general febrile phase followed by signs of vascular leakage, predominantly
73 manifesting as transient kidney failure in HFRS or as life-threatening pulmonary complications in
74 HCPS (7, 8). The pathogenesis of both conditions is immune-mediated, with pronounced activation
75 of immune cells and pro-inflammatory cytokines during acute stages of disease. An intense

76 hantavirus-directed immune response then results in efficient virus clearance in surviving patients
77 (8).

78

79 In contrast to human infection, hantavirus infections are thought to be persistent and asymptomatic
80 in their wildlife reservoirs (6). Each hantavirus species is carried by a specific reservoir host; for
81 example, Puumala orthohantavirus (PUUV), which is present in Europe and causes thousands of
82 human infections annually (9), is carried by the bank vole (*Myodes glareolus*). Based on
83 mitochondrial cytochrome *b* gene sequences different evolutionary lineages of the bank vole with
84 different glacial refugia were defined (10-13). In southern to central Finland the Eastern
85 evolutionary lineage has been identified (13).

86

87 Persistent infections are generally facilitated by host immune tolerance (14), and it has been
88 suggested that activation of regulatory T cells in reservoir hosts could also play a role in enabling
89 hantavirus persistence (15, 16). Deciphering the detailed molecular mechanisms by which reservoir
90 hosts become immune tolerant instead of attempting potentially costly virus clearance could
91 significantly increase our understanding of hantavirus pathogenesis and provide insights into
92 alternative treatment strategies.

93

94 A major issue hindering detailed research on the molecular mechanisms of hantavirus persistence in
95 reservoir hosts has been the absence of a standardized experimental infection system. Two main
96 experimental strategies have been employed by researchers to date: 1) lung homogenates from
97 naturally infected rodent hosts have been used as virus inoculum. However, this method suffers
98 from slowly developing infections and a lack of standardized infection loads (17, 18), and 2)
99 hantaviruses isolated and grown to high titers in cell culture. However, this method has been

100 performed so far almost exclusively using Vero E6 cells, which generates cell culture-adapted
101 viruses with attenuated properties (19-22).

102

103 The purposes of this study are to develop an effective hantavirus experimental wildlife system and
104 to examine the immunologic responses that facilitate persistent hantavirus infections in a reservoir
105 host species. To these means we employed PUUV and its reservoir host, the bank vole. Our first
106 goal was to isolate PUUV from wild bank voles using a host-derived cell line, and to evaluate
107 genotypic changes during the isolation process using next-generation sequencing (NGS).

108 Experimental bank vole infections were then used to compare the viral RNA load and tissue
109 distribution and) of the novel isolate to the wild bank vole-derived PUUV-containing lung
110 homogenate (PUUV-wt), a Vero E6-cell attenuated PUUV-Kazan strain and to PUUV in naturally
111 infected wild voles.

112

113 To examine immunologic responses to PUUV in vole reservoir hosts, we measured T helper cell
114 differentiation pathways by assaying changes in mRNA expression of cytokines and transcription
115 factors (TF) related to T helper (Th) 1-, Th2- and regulatory T (Treg) cell activation. Comparisons
116 were made among voles infected with the novel PUUV isolate, PUUV-Kazan, PUUV-wt and mock
117 infected voles. Since no assays to detect PUUV-specific T cell responses are available for bank
118 voles, the bulk expression levels of T cell-related mRNAs in the spleen were studied both directly
119 in spleens of experimentally infected voles and using single-cell suspensions (splenocytes) from
120 PUUV-negative bank voles after *in vitro* infection with PUUV isolates. To gain further insights into
121 PUUV-specific and non-specific humoral responses to PUUV infections, we additionally measured
122 PUUV nucleocapsid (N)-specific and total immunoglobulin G (IgG) levels in vole blood following
123 infection.

124

125 Results

126

127 *Isolation of PUUV-Suo in epithelial bank vole cells*

128 A renal epithelial cell line Mygla.REC.B of the Western evolutionary lineage of the bank vole was
129 generated to isolate a new PUUV strain named Suonenjoki (PUUV-Suo) from the lungs of a
130 PUUV-infected wild bank vole collected in Suonenjoki, Finland. Based on the detection of PUUV
131 N protein by immunofluorescence assay (Fig. 1), 100% of cells were infected after approximately
132 20 cell passages post initial inoculation with the PUUV-containing lung homogenate. Cell
133 passaging did not influence virus infectivity, as confirmed by consistent detection of progeny
134 viruses in cell culture supernatants at 10^4 fluorescence focus forming units (FFUs)/ml.

135

136 The isolation process resulted in eight nucleotide exchanges when compared to the consensus
137 sequence derived from the lung homogenate (Table 1). Two mutations occurred in non-
138 coding regions, three resulted in amino acid exchanges in the RNA-dependent RNA polymerase
139 (RdRP, L segment), one resulted in an amino acid exchange in the N protein, and two were silent
140 mutations in the glycoprotein precursor (GPC; M segment) and RdRP coding regions. The new
141 virus isolate had a 95-99 % nucleotide sequence identity with partially sequenced PUUV strains
142 from bank voles in Konnevesi and from a fatal human case in Pieksämäki (Supplementary Figs 1-3)
143 (23, 24), both located within 100 km of the vole trapping site in central Finland.

144

145

146 *Replication of PUUV-Suo in Mygla.REC.B cells is resistant to immune stimulation*

147 We hypothesized that the observed persistence of PUUV-Suo in bank vole cells would be a
148 consequence of either impaired host cell antiviral signaling or the ability of the virus to counteract
149 antiviral responses. Therefore, the antiviral function of Mygla.REC.B cells was assessed by relative
150 reverse transcription-real time quantitative polymerase chain reaction (RT-qPCR) quantification of

151 the interferon-inducible myxovirus resistance protein 2 (Mx2) mRNA after exogenous activation of
152 non-infected and infected cells with the toll-like receptor 3 (TLR3) agonist polyI:C or with Sendai
153 virus (SeV, also known as Murine respirovirus). Firstly, supporting the immune competence of
154 MyglA.REC.B cells, a significant upregulation of Mx2 mRNA occurred in non-infected cells with
155 both stimulants (~100-fold and 2000-fold by polyI:C and Sendai virus, respectively, Fig. 2A).
156 Secondly, non- and polyI:C-stimulated PUUV-infected cells showed higher levels of Mx2 mRNA
157 than non-infected cells. PUUV and Sendai virus superinfection had a significant synergistic effect
158 on Mx2 mRNA production (Fig. 2A). These findings suggest that MyglA.REC.B cells are capable
159 of interferon signaling but that persistent PUUV-Suo infection does not impair this pathway.

160

161 We also investigated whether PUUV replication in MyglA.REC.B cells is susceptible to an
162 exogenously induced antiviral state. PUUV-infected cells, non-stimulated or stimulated with
163 polyI:C or Sendai virus, were assessed for PUUV S segment RNA levels by RT-qPCR at 1 and 3
164 days (d) post-stimulation. Neither polyI:C nor Sendai virus stimulation influenced PUUV S
165 segment RNA levels, which increased significantly from days 1 to 3 with and without stimulation
166 (Fig. 2B), indicating that PUUV-Suo infection in MyglA.REC.B cells is not affected by innate
167 immune pathway stimulation.

168

169 *Experimental PUUV-Suo infection resembles natural infection in bank voles*

170 Following experimental infection of bank voles with PUUV-Suo or a mock inoculum (PBS), the
171 amount of PUUV S segment RNA was measured in lungs, spleen, kidney and blood of voles at 3d,
172 and 1, 2 and 5 weeks (wks) post infection (pi). Viral RNA was similarly measured in the urine of
173 experimentally infected voles at 3d, and 1 and 2wks pi. As another control, virus RNA load and
174 RNA distribution in tissues were also assessed at 2 and 5wks pi from voles inoculated with UV-
175 inactivated PUUV-Suo isolate.

176

177 PUUV RNA was detected in all organs of PUUV-Suo infected voles from 3 dpi onwards (Fig. 3A),
178 but not in mock-infected animals nor those inoculated with UV-inactivated virus (data not shown).
179 Highest PUUV RNA levels occurred in the lungs at 3d and 1wk pi and decreased thereafter, with
180 increased levels then observed in the spleen (at 2 and 5wk pi) and kidneys (at 5wk pi). PUUV RNA
181 was detected in the blood of one vole at 3 dpi, suggesting that if PUUV-Suo caused viremia it
182 occurred very early during infection. We also detected PUUV RNA in the urine of all tested PUUV-
183 Suo infected bank voles (n = 7), suggesting virus shedding.

184

185 We further compared the infection dynamics of the PUUV-Suo isolate to a Vero E6 cell-adapted
186 PUUV-Kazan isolate, PUUV-wt and to natural PUUV infections. Low levels of PUUV RNA were
187 detected following infection with PUUV-Kazan, mainly in the lungs of infected voles at 3d pi; after
188 this the virus was efficiently cleared, with viral RNA levels significantly decreased at 1wk pi (Fig.
189 3B). No virus replication was observed for PUUV-wt at 2wk pi, but three out of four voles were
190 positive at 5wk pi (Fig. 3C). At this time point, viral RNA loads were highest in the lungs, followed
191 by the spleen and kidneys. The amount and distribution of PUUV RNA seen in naturally PUUV-
192 infected wild bank voles (with unknown infection timepoint) resembled that seen in PUUV-Suo
193 infected voles during the first week and in voles inoculated with PUUV-wt at 5wk pi (Fig. 3D).

194

195 *PUUV-Suo host cell range resembles natural infections*

196 Both naturally and experimentally infected bank voles were examined by histology and
197 immunohistology for PUUV N protein. The two naturally infected voles did not exhibit any
198 histopathological changes and viral antigen expression was limited in its amount and distribution.
199 Besides endothelial cells in renal glomerula and in some capillaries of the liver, kidney, lungs and
200 heart (Fig. 4A, B), pneumocytes (mainly type II) and macrophages in liver (Kupffer cells) and

201 spleen (red pulp macrophages) were found to be occasionally positive (Fig. 4B). In one animal with
202 a higher number of positive cells, viral antigen was also detected in tubular epithelial cells in the
203 renal cortex and medulla.

204

205 None of the experimentally PUUV-Suo infected voles exhibited histopathological changes (two
206 voles analyzed at each time point). During the early stages of infection (≤ 1 wk pi), the infection
207 pattern was similar to that seen in naturally infected bank voles. Capillary endothelial cells (also in
208 glomerular tufts), pneumocytes and macrophages (Kupffer cells in the liver, pulmonary alveolar
209 macrophages) occasionally exhibited viral antigen expression (Fig. 4C, D). At later time points (2
210 and 5wks pi), viral antigen was only found in macrophages, and almost exclusively in rare
211 macrophages of the splenic red pulp (Fig 4E). No PUUV N protein was detected in PUUV-Kazan-
212 infected voles, consistent with the low level of PUUV RNA in those animals (Fig. 3C).

213

214 Histological examination of the PUUV-wt infected voles at 5wks pi did not reveal any pathological
215 changes. Interestingly, when testing lungs, kidneys and spleen by immunohistochemistry, N protein
216 expression was only detected in one of three animals, and only in the lungs, where a few individual
217 pneumocytes and endothelial cells were positive (Fig. 4F). This animal also showed the highest
218 viral RNA levels in the lungs.

219

220 *PUUV-specific immunoglobulin (Ig) responses are delayed during persistent infections*

221 All PUUV-Suo and PUUV-Kazan infected voles, which were infected for at least one week,
222 seroconverted to produce PUUV-specific Ig but with contrasting kinetics (Fig. 5A; the assay does
223 not discriminate between Ig-subclasses). For PUUV-Suo, specific Ig-titers were low at 1wk but
224 peaked at 2wks pi. This was significantly different from PUUV-Kazan infected voles, in which

225 highest PUUV-specific Ig titers were detected at 1wk pi. For PUUV-wt, only one of three infected
226 voles had seroconverted at 3 wks pi (Fig. 5A).

227

228 Next we measured total IgG levels in the blood of experimentally infected voles. Compared to
229 mock-infected animals at 2wks pi, voles infected with PUUV-Suo showed elevated total IgG levels,
230 while no difference was observed between voles infected with the PUUV-Kazan or the mock
231 inoculate at 1wk and 2wks pi (Fig. 5B). The two PUUV-wt infected voles analyzed had elevated
232 total IgG levels when compared to mock-infected voles (Fig. 5B), despite only one showing a
233 PUUV-specific response (Fig. 5A).

234

235 To gain further insights into whether total IgG levels were comprised predominantly of PUUV-
236 specific responses in experimentally infected voles, PUUV-specific Ig and total IgG titers were
237 assessed also in naturally PUUV-infected voles. Despite detecting PUUV-specific responses at
238 levels comparable to experimentally PUUV-Suo infected voles, total IgG in naturally infected voles
239 was not elevated when compared to non-infected voles (Fig. 5C), suggesting that the acute total IgG
240 response towards PUUV-Suo infection involves PUUV non-specific IgG.

241

242 *Minor effects of PUUV infection on splenic T cell related gene expression*

243 To assess the potential effects of experimental PUUV infection on T cell differentiation, we assayed
244 pre-selected splenic T cell related mRNAs in mock-, PUUV-Suo and PUUV-wt infected voles for
245 transcriptional regulation (the same animals as those examined by PUUV-specific RT-PCR in Fig.
246 3). Transcription levels of cytokines associated with Th1, Th2 or Treg cell activation (interferon
247 {IFN}- γ , interleukin {IL}-4, IL-10 or transforming growth factor {TGF}- β) did not differ
248 significantly between PUUV- and mock-infected voles (Fig. 6). Of key transcription factors for
249 Th1, Th2 and Treg pathways (Tbet, Gata binding protein 3 {Gata3} or Forkhead box P3 {FoxP3},

250 respectively), FoxP3 expression was significantly lower in PUUV-wt infected than in mock-
251 infected voles at 5 wks dpi. A similar trend, although not statistically significant, was observed for
252 PUUV-Suo at 2 and 5 wks dpi. Another significant change in immune gene regulation was
253 represented by the upregulation of Mx2 gene expression in response to PUUV-Suo infection in
254 comparison to mock infection at 5wks pi (Fig. 6B). A trend towards higher Mx2 mRNA levels in
255 response to PUUV-wt was also observed at the same time point (Fig. 6C).

256

257 *PUUV-Suo promotes immunoregulation via cytokine IL-10*

258 Splenocytes isolated from PUUV-negative wild-caught bank voles were inoculated *in vitro* with
259 PUUV-Suo, PUUV-Kazan and UV-inactivated PUUV-Suo and assessed for changes in T cell-
260 related gene expression. IFN- γ mRNA levels were higher in PUUV-Kazan infected splenocytes
261 than in PUUV-Suo infected splenocytes (~11-fold vs. ~3-fold increase in comparison to non-
262 infected controls, respectively; Fig. 7). In contrast, IL-10 mRNA expression was significantly
263 upregulated in response to PUUV-Suo when compared to PUUV-Kazan and UV-inactivated
264 PUUV-Suo (~6-fold vs. 2-3-fold increase in comparison to non-infected controls, respectively). No
265 other significant differences in transcription levels of other genes (TGF- β , Mx2, T-bet, Gata3) were
266 observed. However, we were unable to measure IL-4 and FoxP3 mRNA, likely due to the generally
267 lower RNA levels in single-cell suspensions than in tissue samples. The PUUV-specific RT-PCR
268 confirmed that live viruses (PUUV-Suo and Kazan), but not UV-inactivated viruses replicated in
269 bank vole splenocytes at a comparable level; however the level of infection varied significantly
270 among splenocyte donors (data not shown).

271

272 Discussion

273 In this study we established and validated an experimental hantavirus rodent reservoir model. Using
274 a virus newly isolated on a bank vole cell line, we conducted standardized experimental *in vivo* and

275 *in vitro* infections to examine immune responses at cellular and organismic levels, gaining insight
276 into the mechanisms of PUUV persistence in the reservoir host. These experiments revealed that
277 activation of the immunoregulatory cytokine IL-10 differentiates wild-type PUUV from attenuated
278 isolates, which is likely to be a key factor in hantavirus persistence. Experimentally infected voles
279 also exhibited a delayed PUUV-specific humoral response, suggesting that PUUV has developed
280 mechanisms to evade host immune recognition.

281

282 The isolation of hantaviruses in cell culture is rare and has so far typically been achieved using
283 interferon type 1-deficient cells (Vero E6 cells) derived from African green monkeys (25), which
284 result in attenuated wild-type properties of the virus (19-22). The PUUV-Suo strain is the first
285 hantavirus isolated on a cell line of the respective natural reservoir host. The isolation process of
286 PUUV-Suo in bank vole renal epithelial cells resulted in a significantly lower nucleotide
287 substitution frequency (0.06 %) when compared to a PUUV-Kazan strain on Vero E6 cells (21, 26)
288 and persistent cell culture infections that were refractory to exogenous stimulation of innate
289 immune pathways. Importantly, the PUUV-Suo isolate caused persistent vole infections without
290 obvious pathological effects. Target cell patterns and viral RNA loads, particularly during the early
291 stages of infection, were consistent with those seen in naturally infected voles and were
292 considerably different from experimental infections conducted with the attenuated PUUV-Kazan
293 isolate. The MyGla.REC.B cell line used to isolate PUUV-Suo is derived from the Western
294 evolutionary lineage, which is different from the Eastern bank vole lineage present in central
295 Finland (27) and used as the source and target of PUUV-Suo isolate. The efficient replication of the
296 Eastern bank vole lineage-derived PUUV-Suo strain in Western lineage bank vole-derived cell line
297 is in line with results from a field study in Germany demonstrating transmission of Western lineage-
298 associated PUUV to sympatrically occurring bank voles of the Eastern and Carpathian lineages
299 (28). Further *in vitro* studies are needed to determine whether heterologous bank voles evolutionary

300 lineages could support PUUV isolation and/or infection. These future investigations may profit
301 from the recent establishment of permanent bank vole cell lines from different evolutionary lineages
302 of the bank vole (29).

303

304 The viral target cell pattern observed for PUUV-Suo is similar to, but less widespread than in
305 weanling and suckling bank voles that were inoculated with lung homogenates from PUUV-positive
306 bank voles (17). Experimental infections conducted with Sin Nombre orthohantavirus (SNV) in
307 deer mice yielded comparable, though apparently more intense viral antigen expression (18). In
308 naturally infected deer mice, however, only pulmonary and cardiac vascular endothelial cells were
309 found to be positive, with an expression intensity that resembled that observed in the present study
310 (30). Interestingly, only PUUV-Suo appears to infect renal tubular epithelial cells, which would
311 account for more intense virus shedding in urine. This could reflect different primary transmission
312 routes for PUUV and SNV, with PUUV suggested to be shed more in urine and SNV in the saliva
313 (6).

314

315 In support of the ability of PUUV to evade T cell immune responses, we detected no significant
316 upregulation in mRNA expression (including transcription factors Tbet, Gata3, FoxP3 and
317 cytokines IFN- γ , IL-4, IL-10 and TGF- β) in the spleens of bank voles infected with PUUV-Suo or
318 wild PUUV-containing lung homogenates (PUUV-wt). Instead, a decrease in FoxP3 levels in
319 PUUV-wt infected voles is contrary to the suggested role of the Tregs in mediating virus
320 persistence, as detected in other experimental hantavirus-reservoir host interactions (31, 32). These
321 discrepant results could be explained by differences in experimental setups. While we measured
322 bulk FoxP3 mRNA levels in the spleen, others have looked either at splenic antigen-specific (31) or
323 lung-resident (32) Treg responses. It is possible that the decrease in FoxP3 mRNA and associated
324 Treg levels in the spleen reflects the recruitment of these cells into peripheral sites of infection in

325 our experimental setup. However, inherent differences in the persistence mechanisms among PUUV
326 and other hantaviruses cannot be ruled out and needs to be further evaluated.

327

328 An interesting finding was that PUUV-Suo induced IL-10 mRNA expression in bank vole
329 splenocyte cultures. This was not observed with attenuated PUUV-Kazan, which instead induced
330 IFN- γ . IL-10 is well-known for its ability to hinder pro-inflammatory responses (33) and could
331 explain the lack of IFN- γ induction in PUUV-Suo infected splenocytes. The fact that we did not
332 observe elevated IL-10 mRNA levels in the spleen of PUUV-Suo (or PUUV-wt) infected voles
333 could be explained by a higher multiplicity of infection (m.o.i.) achieved in an *in vitro* environment
334 using cultured splenocytes. It is also possible that a potential increase in IL-10 transcription does
335 occur in the spleen of infected voles, but is short-lived and thus difficult to observe using samples
336 collected with several day intervals.

337

338 The increase in IL-10 expression due to PUUV in bank vole splenocytes is not surprising given the
339 established role of this cytokine in promoting chronic infections (34, 35). Lymphocytic
340 choriomeningitis virus (LCMV) infection in mice is the best-studied non-human model for
341 persistent virus infections. For this mammarenavirus, IL-10 blocking experiments have been shown
342 to lead to virus clearance, demonstrating the key role of IL-10 in driving persistent infections (36-
343 38). The immunomodulatory effects of IL-10 are exerted through antigen presenting cells (APCs)
344 which, in response to IL-10, inhibit effector Th1 cell function and downregulate inflammation (33).
345 Our results demonstrating early induction of IL-10 in PUUV-infected splenocytes together with
346 findings of virus-infected macrophages *in vivo* indicate a similar scenario in PUUV-infected bank
347 voles; virus reverts early macrophage responses from a typical inflammatory milieu towards
348 immunomodulation with subsequent inhibition of IFN- γ expression, Th1 cell activation and virus
349 clearance. In fact, corroborating the differential response between human and reservoir host APCs

350 to hantavirus infection, human dendritic cells have been shown to decrease IL-10 expression while
351 elevating pro-inflammatory responses when in contact with Andes orthohantavirus-infected
352 endothelial cells (39).

353

354 Inadequate humoral responses have not been previously considered as a mechanism of persistent
355 hantavirus infections in reservoir hosts. This is due to observations of strong virus-specific IgG
356 responses and high neutralizing antibody titers in hantavirus infected wild rodents (36-38).
357 Therefore, our finding that PUUV-specific Ig responses were significantly delayed in voles infected
358 with PUUV-Suo was unexpected, as was the lack of seroconversion in two of three bank voles
359 infected with PUUV-wt. Interestingly, an acute increase in total IgG levels that cannot be solely
360 explained by the PUUV-specific Ig response was observed for PUUV-Suo and PUUV-wt. An
361 increase in total Ig levels, i.e. hypergammaglobulinemia, is strongly associated with other persistent
362 viral infections in both humans and rodents (40-42). The mechanisms by which
363 hypergammaglobulinemia facilitates persistent infections are currently unknown but could involve
364 virus-mediated polyclonal activation of B cells and subsequent exhaustion of specific B cell
365 responses (41). These could allow sufficient time for the virus to establish systemic infection that,
366 despite later robust virus-specific humoral responses, the host is unable to clear.

367

368 Our results show that a hantavirus isolated using allogeneic reservoir host cells may retain wild-type
369 characteristics better than a Vero E6-adapted virus, and should be considered as the preferred
370 method for future hantavirus isolations and experimental infections. Using these techniques, we
371 demonstrated that immune regulatory mechanisms are likely to facilitate persistent PUUV
372 infections in bank voles, which provide insights into other difficult to study wildlife-hantavirus
373 systems and may help guide novel therapeutic strategies for human infections.

374

375 Materials and methods

376 *Ethics statement*

377 All vole trapping and experimental procedures performed in Finland were approved the Animal
378 Experimental Board of Finland (license number ESAVI/6935/04.10.07/2016). Wild bank voles
379 representing the Eastern evolutionary lineage were captured in Ugglan live traps around central
380 Finland. To generate the Mygla.REC.B cell line, an adult bank vole of the Western evolutionary
381 lineage was taken from the breeding colony at the Friedrich-Loeffler-Institut, Greifswald-Insel
382 Riems, Germany.

383

384 *Establishment of the permanent cell line*

385 The bank vole from the breeding colony was anaesthetized with isoflurane and euthanized by using
386 CO₂. During dissection the kidney was removed and shipped in transport medium on ice to the
387 Institute of Virology in Bonn, where the cell line was established. Briefly, the kidney was washed in
388 sterile PBS and minced with a blade. Tissue was resolved with 37°C Dulbecco's minimum essential
389 medium-high glucose (DMEM; Sigma Aldrich) supplemented with 10 % inactivated fetal calf
390 serum (FCS), 100 IU/ml Penicillin, 100 µg/ml Streptomycin, 2mM L-glutamine and a mix of non-
391 essential amino acids (Sigma Aldrich), containing in addition 1% Ofloxain (Tarivid), and seeded
392 into 6-well plates. After 5-7 days, primary cell growth could be observed, and medium was
393 renewed. Primary cells were immortalized when confluency of 40-50% was reached by using a
394 lentiviral system carrying the large T antigen of SV40, as described previously (43). Once an
395 increase in cell proliferation was observed, cells were further passaged and cryoconserved.

396

397 *Virus isolation and propagation*

398 For virus isolation, a serologically confirmed PUUV-infected bank vole from Suonenjoki, Finland
399 and representing Eastern evolutionary lineage, was euthanized and lung samples were collected and

400 frozen at -70 °C. The frozen lungs were then homogenized with a mortar and pestle in 1 ml of
401 phosphate-buffered saline (PBS) over dry ice. After thawing, 500 µl of the homogenate was
402 incubated for 1 h with semi-confluent Mygla.REC.B cells, which were grown similarly as described
403 above. Mygla.REC.B cells were passaged at 3-day intervals until 100% of cells were found to be
404 infected with PUUV via immunofluorescence assay as described below. We named the newly
405 isolated hantavirus PUUV-Suonenjoki (PUUV-Suo) due to the geographical location of the bank
406 vole trapping site.

407

408 An attenuated PUUV isolate, PUUV-Kazan strain (21), which was previously isolated and
409 propagated in Vero E6 cells (green monkey kidney epithelial cell line; ATCC no. CRL-1586),
410 served as comparison for experimental PUUV-Suo infections. PUUV-Kazan was grown in Vero E6
411 cells using Minimum Essential Medium (MEM; Sigma Aldrich) and supplemented with 10 %
412 inactivated FCS, 100 IU/ml of Penicillin and 100 µg/ml of Streptomycin and 2mM of L-glutamine.
413 Both PUUV-Suo and PUUV-Kazan strains were purified from supernatants of infected cells
414 through a 30 % sucrose cushion by ultracentrifugation and resuspended in corresponding growth
415 medium. Virus titers were measured by incubating diluted virus stocks with Vero E6 cells for 24 h
416 at 37 °C, followed by acetone fixation and staining with a rabbit polyclonal antibody specific for
417 PUUV N protein and AlexaFluor488-conjugated secondary antibody. Fluorescent focus-forming
418 units (FFFU)/ml were counted under a UV microscope (Zeiss). UV inactivation of virus
419 preparations was carried out using a Stratalinker UV crosslinker (300,000 µJ/cm²).

420

421 *Evolutionary lineage determination of bank voles and cell line*

422 The lineage determination was based on an established protocol using amplification of a partial
423 cytochrome *b* gene segment and comparison to prototype sequences of the various bank vole
424 lineages (28, 44)

425

426 *Next-generation sequencing*

427 Prior to RNA extraction, the bank vole lung homogenate and Mygla.REC.B cell culture supernatant
428 were treated with a cocktail of micrococcal nuclease (New England BioLabs) and benzonase
429 (Millipore) for 1 h at +37°C. RNA was extracted with Trizol and ribosomal RNA was removed
430 using a NEBNext rRNA depletion kit (New England BioLabs), according to manufacturer
431 instructions. The sequencing library was prepared using a NEBNext Ultra II RNA library prep kit
432 (New England BioLabs) and quantified using a NEBNext Library Quant kit for Illumina (New
433 England BioLabs). Pooled libraries were then sequenced on a MiSeq platform (Illumina) using a
434 MiSeq v2 reagent kit with 150 bp paired-end reads. Raw sequence reads were trimmed and low-
435 quality (quality score <30) and short (<50 nt) sequences were removed using Trimmomatic (45).
436 Thereafter, *de novo* assembly was conducted using MegaHit, followed by re-assembly against the
437 *de-novo* assembled consensus sequences using BWA-MEM implemented in SAMTools version 1.8
438 (46).

439

440 *Phylogenetic analysis*

441 The complete L-, M- and S-segment sequences were downloaded from GenBank and aligned using
442 MUSCLE program package (47). Substitution model was estimated using jModeltest2 (48).
443 Phylogenetic trees were then constructed using the Bayesian Markov chain Monte Carlo (MCMC)
444 method, implemented in Mr Bayes version 3.2 (49) using a GTR-G-I model of substitution with 2
445 independent runs and 4 chains per run. The analysis was run for 500 000 states and sampled every
446 5,000 steps.

447

448 *Cell cultures*

449 PUUV-infected cells, grown on a black 96-well plate (Nunc), were detected by
450 immunofluorescence assay after acetone fixation using a polyclonal rabbit anti-PUUN serum
451 diluted 1:1000 in PBS followed by AlexaFluor488-conjugated donkey anti-rabbit secondary
452 antibody (Thermo Scientific). Nuclei were stained using Hoechst 33420 diluted 1:5000 in PBS.
453 Images were taken using a Leica TCS SP8 X confocal microscope (Biomedicum Imaging Unit core
454 facility, Medicum, University of Helsinki).

455

456 Non- and PUUV-Suo infected Mygla.REC.B cells were exogenously stimulated with TLR-3 ligand
457 polyI:C (10 µg/ml, Sigma Aldrich) or Sendai virus (multiplicity of infection 1; received from Prof.
458 Ilkka Julkunen; National Institute of Health and Welfare, Helsinki, Finland). Cells were collected at
459 1 and 3-d post-treatment and RNA expression analysis was performed as described below. All
460 experiments were conducted in duplicate.

461

462 *Experimental infections*

463 Wild-captured voles from PUUV endemic region in Central Finland were transported to the
464 University of Helsinki BSL-3 facility and acclimatized to individually ventilated biocontainment
465 cages (ISOcage, Scanbur) for two days with *ad libitum* water and food (rodent pellets and small
466 pieces of turnip every second day). Prior to any treatments, a small blood sample was collected
467 from the retro-orbital sinus of each animal, and their PUUV infection status was determined by
468 immunofluorescence assay (see below).

469

470 PUUV seronegative voles were divided into five treatment groups and subcutaneously injected
471 with: 1) 100 µl of PBS (mock infection; n = 2 per time point), 2) 10,000 FFFUs of PUUV-Suo (n =
472 4 per time point), 3) UV-inactivated PUUV-Suo (n = 2 per time point), 4) 10,000 FFFUs of PUUV-
473 Kazan (n = 2-3 per time point), or 5) a pooled homogenate prepared from the lungs of five PUUV-

474 seropositive wild bank voles (PUUV-wt; n = 4 per time point). A total of 43 individual voles were
475 used across the different time points and experimental treatments. The lung homogenate was
476 obtained after grinding frozen lung tissue from naturally PUUV-infected voles (confirmed by
477 PUUV-specific RT-PCR) with mortar and pestle in 1 ml PBS on dry ice. The PUUV-Suo and
478 PUUV-wt were derived from voles captured from the same geographical area in Central Finland
479 and together with voles used for experimental infections represent the Eastern bank vole
480 evolutionary lineage. Blood samples from all treatment groups were collected from the retro-orbital
481 sinus at 1wk intervals pi. Urine samples were collected from PUUV-Suo infected voles at 3d, and 1
482 and 2wks pi. Voles were sacrificed using isoflurane anesthesia, followed by cervical dislocation at
483 3d pi and 1 (7d), 2 (14-16d), and 5wks (35-38d) pi to collect samples for viral RNA load and
484 distribution analyses, immunohistology and gene expression assays.

485

486 *Virus quantification*

487 Following euthanasia and dissections, RNA extractions on bank vole tissues and urine were
488 performed using Trisure (Bioline) according to the manufacturers' instructions, with 10 µg/ml
489 glycogen as carrier. RNA was directly subjected to PUUV S-segment RT-qPCR analysis based on a
490 previously described protocol (50), with TaqMan fast virus 1-step master mix (Thermo scientific)
491 using AriaMx instrumentation (Agilent).

492

493 *Histological and immunohistochemistry examinations*

494 Two wild-trapped, naturally PUUV-infected adult bank voles were dissected and samples from
495 brain, heart, lung, liver, kidneys and spleen were fixed in 10% neutral-buffered formalin. Similarly,
496 lung, liver, spleen and kidney samples were collected and formalin-fixed from each two PUUV-Suo
497 infected voles euthanized at 3d, 1wk, 2wks, and 5wks pi and two PUUV-wt infected bank voles
498 euthanized at 5wks pi. The latter two voles were initially frozen at -80 °C, and tissue fixation was

499 achieved by slowly thawing the organ samples in ice-cold formalin. After 4-7 days in formalin,
500 tissue specimens were transferred to 70% ethanol, trimmed and routinely paraffin wax embedded.
501 Consecutive sections (3-5 μm) were prepared and routinely stained with hematoxylin-eosin (HE) or
502 subjected to immunohistology for the detection of PUUV N antigen in tissues.
503
504 Anti-PUUV N protein antiserum was generated by immunization of a single rabbit with PUUV N
505 protein produced via baculovirus-mediated expression. The same batch of PUUV N protein was
506 used in an earlier diagnostic study (51) and the immunization by BioGenes GmbH (Berlin,
507 Germany) was as described in (52). Immunohistology was performed in an autostainer (Dako) using
508 the custom-made rabbit polyclonal antiserum and the horseradish peroxidase (HRP) method.
509 Briefly, sections were deparaffinized and rehydrated through graded alcohol. Antigen retrieval was
510 achieved by 20 min incubation in citrate buffer (pH 6.0) at 98°C in a pressure cooker. This was
511 followed by incubation with the primary antibody (diluted 1:1,000 in dilution buffer; Dako) for 60
512 min at room temperature (RT), a 10 min incubation at room temperature (RT) with peroxidase
513 blocking buffer (Dako) and a 30 min incubation at RT with Envision+System HRP Rabbit (Dako).
514 The reaction was visualized with diaminobenzidin (DAB; Dako). After counterstaining with
515 hematoxylin for 2 sec, sections were dehydrated and placed on a coverslip with Tissue-Tek Film
516 (Sysmex). A formalin-fixed and paraffin embedded pellet of Vero E6 cells infected with PUUV for
517 14 days served as a positive control (infected cells exhibit a finely granular to coarse cytoplasmic
518 staining).

519

520 *PUUV-specific and total IgG assays*

521 Immunofluorescence assays, using PUUV Sotkamo strain-infected Vero E6 cells fixed to
522 microscope slides with acetone, were used to evaluate PUUV-specific Ig in bank vole blood (53).
523 After incubating slides with blood diluted in PBS (1:10), bound Igs were detected with Fluorescein

524 isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig antibody (Dako Cytomation). To quantify
525 total IgG levels, an enzyme-linked immunosorbent assay kit detecting mouse IgG (Mabtech) was
526 used, replacing the mouse IgG standard with bank vole IgG 4G2 (specific for the PUUV
527 glycoprotein) (54). One PUUV-wt infected vole had high total IgG levels already at the beginning
528 of the experiment and was removed from the analysis.

529

530 *Splenocyte stimulations*

531 To directly assess the effect of PUUV infection on immune cells, we extracted single cell
532 suspensions from the homogenized spleens of 3 wild-caught PUUV-seronegative bank voles by
533 washing the homogenate through 70 μ M cell strainers (Sigma Aldrich) with 10 ml PBS. After
534 pelleting through centrifugation, cells were incubated in 3 ml of Ammonium-Chloride-Potassium
535 (ACK) lysing solution to lyse the red blood cells, washed in PBS and frozen in CryoStor CS10
536 (Sigma Aldrich) at -130°C until further investigation. After thawing and washing in PBS,
537 splenocytes were suspended in RPMI-1640 supplemented with 10% inactivated FCS, 100 IU/ml of
538 Penicillin, 100 μ g/ml of Streptomycin, 2mM of L-glutamine and 25 mM Hepes pH 7.4. For
539 stimulations, 1 million cells were either: 1) untreated, 2) treated with 20 μ g/ml lipopolysaccharide
540 (LPS), or 3) infected with virus at a m.o.i. of 0.01 in 250 μ l of medium. Cells were collected after 3
541 days of incubation at 37 °C and subjected to RT-qPCR as described below. As an indicator of
542 splenocyte viability after thawing, we used only splenocytes that showed a minimum of 5-fold
543 increase in IFN- γ mRNA levels in response to LPS when compared to untreated, non-infected
544 controls.

545

546 *Transcription factor and cytokine assays*

547 To analyze bank vole gene expression profiles, RNA extracted from spleens and cell cultures was
548 reverse transcribed to complementary DNA (cDNA) using random hexamers and RevertAid reverse

549 transcriptase (Thermo Scientific). Relative quantitative PCR was performed with Maxima SYBR
550 Green master mix (Thermo Scientific) using AriaMx instrumentation (Agilent).
551
552 Primers used to measure the mRNAs levels of the interferon-inducible gene Mx2, a marker of
553 innate immunity activation towards virus infection (55), and actin, used for normalization of mRNA
554 levels between samples, are described in (56). Primers used to amplify bank vole mRNAs of Tbet,
555 Gata3, FoxP3, IFN- γ , TGF- β , and IL-10 were originally designed for field voles and are described
556 in (57). Primers to detect IL-4 mRNA (Forward: 5'-GCT CTG CCT TCT AGC ATG TAC T-3' and
557 Reverse: 5'-TGC ATG GCG TCC CTT TTT CT-3') were designed based on the annotated mRNA
558 sequence of the prairie vole (*Microtus ochrogaster*). All primers were validated for the
559 corresponding bank vole sequence based on the expected amplicon sizes in agarose gels. Samples
560 were excluded from further analysis when the PCR did not produce the expected single peak
561 melting curve. Fold changes of individual mRNA expression levels relative to mock-infected
562 controls were performed by the comparative CT method (58).

563

564 *Statistical analyses*

565 Statistical differences between groups were assessed by one- or two-way ANOVA with Dunnett's
566 or Sivak's multiple comparison tests as indicated in the figure legends. Analyses were conducted
567 using GraphPad Prism version 8.1.2.

568

569 *Acknowledgements*

570 We thank Sanna Mäki, Irina Suomalainen (University of Helsinki) and laboratory technicians in the
571 Histology Laboratory at the University of Zurich for technical assistance. The authors would like to
572 thank Markus Keller and Susanne Röhrs for provision of the bank vole, Matthias Lenk for
573 dissection and provision of the kidney tissue for establishment of the Mygla.REC.B cell line, Dörte

574 Kaufmann, Florian Binder and Stephan Drewes for determination of the evolutionary lineage by
575 cytochrome *b* gene sequencing, and Stephan Drewes for helpful comments. This work was
576 financially supported by the Academy of Finland (Grant 1275597 to TS, 1308613 to JH) and the
577 Finnish Cultural Foundation (grant to KMF). The work of RGU (RoBoPub consortium) was
578 supported by the Federal Ministry of Education and Research (BMBF) within the Research
579 Network Zoonotic Infectious Diseases (FKZ 01KI1721A RGU). The work of IE (EpiZell) was
580 funded by Federal Ministry of Education and Research (BMBF) through the National Research
581 Platform for Zoonosis Research (FKZ 01KI1308).

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- 735
- 736

737 **Supplementary figures**

738

739 Supplementary Figure 1. Phylogenetic analysis of the complete PUUV-Suo S segment. Analysis of
740 both the original bank vole lung (lung homogenate) and Mygla.REC.B.-isolated (cell culture
741 supernatant) are included and marked as red.

742 Supplementary Figure 2. Phylogenetic analysis of the complete PUUV-Suo M segment. Analysis of
743 both the original bank vole lung (lung homogenate) and Mygla.REC.B.-isolated (cell culture
744 supernatant) are included and marked as red.

745 Supplementary Figure 3. Phylogenetic analysis of the complete PUUV-Suo L segment. Analysis of
746 both the original bank vole lung (lung homogenate) and Mygla.REC.B.-isolated (cell culture
747 supernatant) are included and marked as red.

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755 Figures

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757 Figure 1. Immunofluorescence image showing successful isolation of PUUV-Suo in a bank vole
758 cell line. Non-infected (mock) or PUUV-Suo infected bank vole kidney epithelial cells
759 (Mygla.REC.B) were stained for nuclei (DNA) using Hoechst 33420 (blue) and for PUUV
760 nucleocapsid protein (PUUN) using PUUN-specific rabbit polyclonal antibody followed by
761 AlexaFluor488-conjugated secondary antibody (green).

762

763 Figure 2. Interaction between PUUV-Suo infection and innate immune pathway stimulation in
764 Mygla.REC.B cells. **(A)** Non-infected (mock) or PUUV-Suo infected Mygla.REC.B cells were
765 either non-stimulated (No stim), stimulated with polyI:C or infected with Sendai virus (Sendai) for
766 1 day. RNA was isolated from cells and subjected to relative quantification of Mx2 mRNA using
767 RT-qPCR. **(B)** PUUV-Suo infected Mygla.REC.B cells were either non-stimulated, stimulated with
768 polyI:C or infected with Sendai virus for 1 and 3 days. RNA was isolated from cells and subjected
769 to relative quantification of PUUV S segment RNA using RT-qPCR. ** ($p < 0.01$) or **** ($p <$
770 0.001). ns, not significant. Bars represent mean \pm standard deviation ($n = 2$).

771

772 Figure 3. Viral RNA load analysis of experimentally and naturally infected bank voles. The lungs,
773 spleen and kidneys of bank voles infected with PUUV-Suo **(A, n = 4)**, PUUV-Kazan **(B, n = 3)** or
774 PUUV-wt **(C, n = 3)** were collected at indicated times post infection to quantify PUUV copy
775 numbers by PUUV S segment-specific qPCR. In addition, seropositive naturally PUUV-infected
776 bank voles with unknown infection timepoints were included as reference **(D, n = 27-38)**. Bars
777 represent mean + standard deviation.

778

779 Figure 4. PUUV target cell analysis in experimental and natural infections. **(A)** Kidneys of naturally
780 infected bank voles: Viral antigen expression is apparent in occasional glomerular endothelial cells
781 (arrowheads) and occasional vascular endothelial cells (inset: arrows). **(B)** Lungs and spleen of
782 naturally infected bank voles: A variable number of vascular/capillary endothelial cells (arrows) and
783 pneumocytes (arrowheads) express viral antigen in the lungs. Very few cells in the splenic red pulp,
784 consistent with macrophages, are also found to express viral antigen (inset: arrow). **(C)** Kidney of
785 PUUV-Suo infected bank vole at 1 wk pi show scattered glomerular endothelial cells with viral
786 antigen expression. **(D)** PUUV-Suo infected vole at 3 d exhibit occasional positive alveolar
787 epithelial cells in the lungs (arrowhead, also in top inset), and scattered positive Kupffer cells in the
788 liver (bottom inset: arrow). **(E)** The spleen of PUUV-Suo infected voles at 2 wks pi show very
789 limited viral antigen expression, which is restricted to individual macrophages, for example in the
790 splenic red pulp (arrowheads). **(F)** The lungs of PUUV-wt infected bank vole at 5 wks pi show viral
791 antigen expression restricted to occasional vascular endothelial cells (left, arrow) and scattered
792 pneumocytes in the lungs (right, arrowhead). The images are representative of two voles analyzed
793 by immunohistochemistry for PUUV N protein expression in naturally infected voles and at each
794 time point of PUUV-Suo or PUUV-wt infections. Haematoxylin counterstain. Bars = 10 μ m.

795

796 Figure 5. Assessment of PUUV-specific Ig and total IgG responses in blood of experimentally and
797 naturally infected bank voles. **(A)** PUUV-specific Ig titers in blood of PUUV-Suo (n = 4), PUUV-
798 Kazan (n = 3-5) and PUUV-wt (n = 3) infected voles at indicated time points. **(B)** Total IgG levels
799 in blood of PUUV-Suo (n = 4-12), PUUV-Kazan (n = 2) and PUUV-wt (n = 2) infected voles at
800 indicated time points. As a comparison, blood of mock- (n = 2-8) and UV-inactivated PUUV-Suo (n
801 = 2) infected voles were also assessed at the indicated time points. **(C)** PUUV-specific Ig titers and
802 total IgG levels in blood of naturally PUUV-infected or non-infected bank voles with unknown

803 infection timepoints (n = 8). Bars represent mean +/- standard deviation. * indicates statistically
804 significant difference (p < 0.05).

805

806 Figure 6. Comparison of T-cell related transcription factor and cytokine mRNA levels in
807 experimentally PUUV-infected voles. The spleens of mock (**A**, n = 2), PUUV-Suo (**B**, n = 4), and
808 PUUV-wt (**C**, n = 3) infected voles were subjected to relative mRNA expression level analysis by
809 RT-qPCR at indicated time points. Statistically significant differences were assessed separately for
810 each gene between mock- and PUUV-infected voles. * (p < 0.05). Bars represent mean +/- standard
811 deviation.

812

813 Figure 7. Comparison of cytokine mRNA responses in bank vole splenocytes infected with PUUV-
814 Suo and PUUV-Kazan isolates. Splenocytes were isolated from PUUV-negative bank voles and
815 either infected with UV-inactivated or live PUUV isolates or not infected (n = 3 for PUUV-Suo; n
816 = 2 for PUUV-Kazan; multiplicity of infection 0.01 for both). Quantification of mRNAs was
817 conducted relative to non-infected splenocytes using RT-qPCR. * (p < 0.05) or ** (p < 0.01). Bars
818 represent mean +/- standard deviation.

819

820

821 Table 1. Comparison between the consensus nucleotide sequences and amino acid sequences of the
 822 PUUV-Suo strain at the pre-isolation stage in the lungs of a naturally infected bank vole and after
 823 isolation in Mygla.REC.B cells.

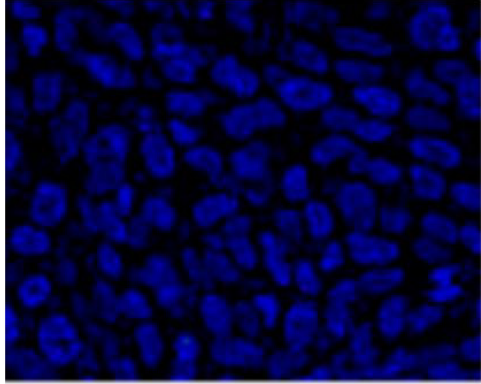
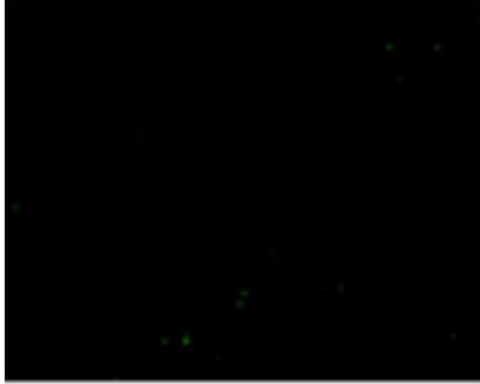
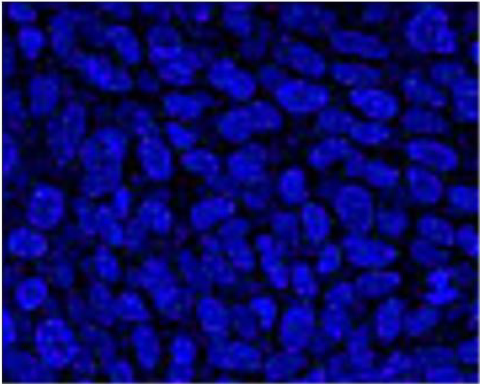
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Segment	Nucleotide position in anti-genomic orientation	Nucleotide in original virus	Nucleotide in isolate	Amino acid in original virus	Amino acid in isolate	Codon position
L	66	A	G	no change		3rd
	411	G	A	Methionine	Isoleucine	3rd
	5700	A	G	Isoleucine	Methionine	3rd
	5942	C	U	Alanine	Valine	2nd
M	89	U	C	no change		1st
	3544	U	C			non-coding
S	136	G	U	Valine	Leucine	1st
	1625	C	U			non-coding

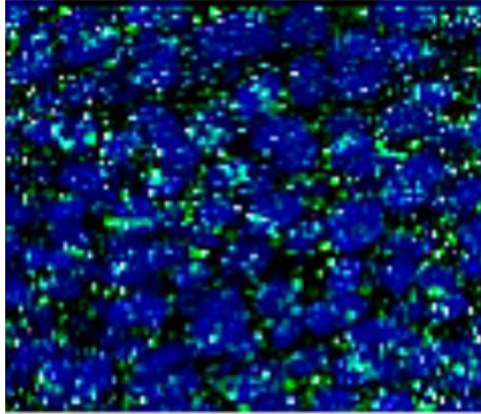
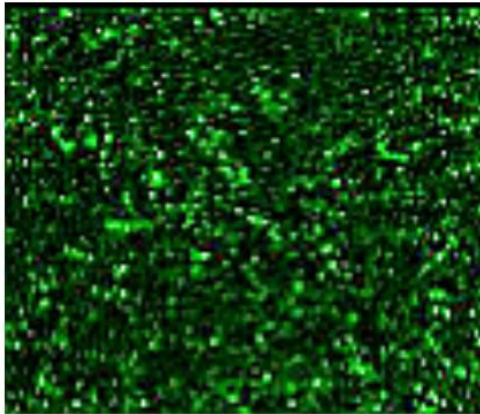
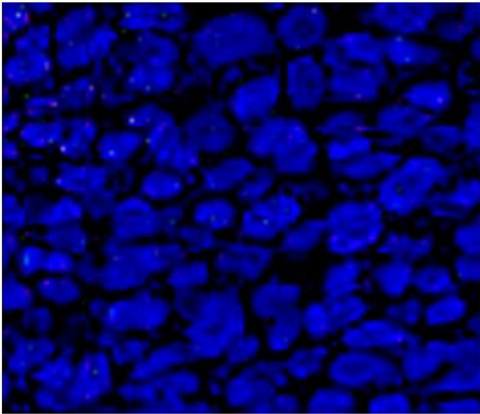
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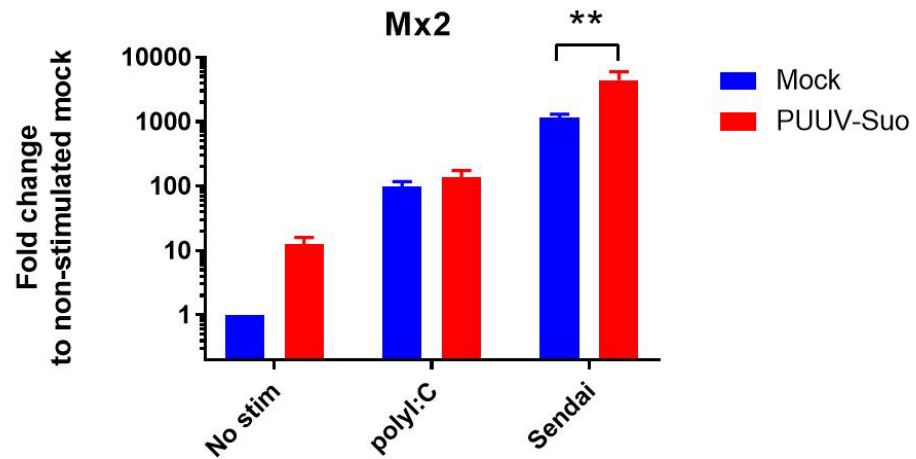
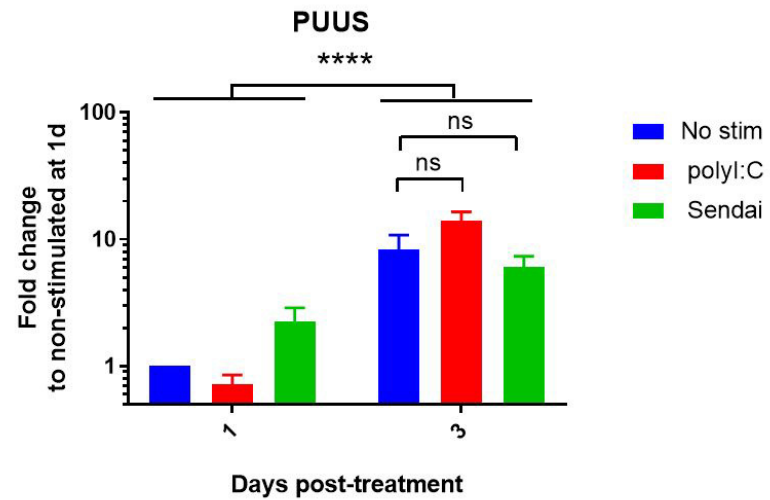
DNA **PUUN** **Overlay**

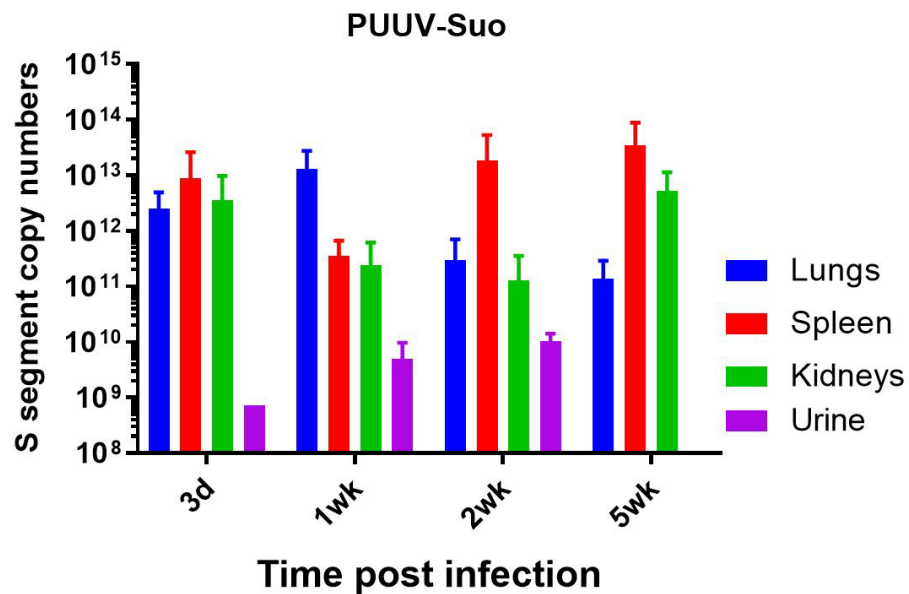
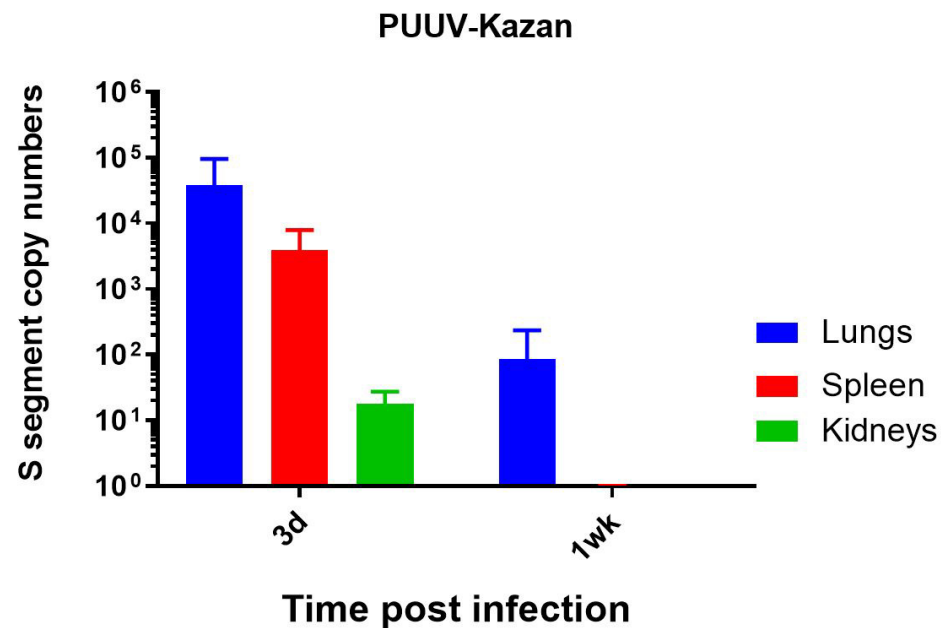
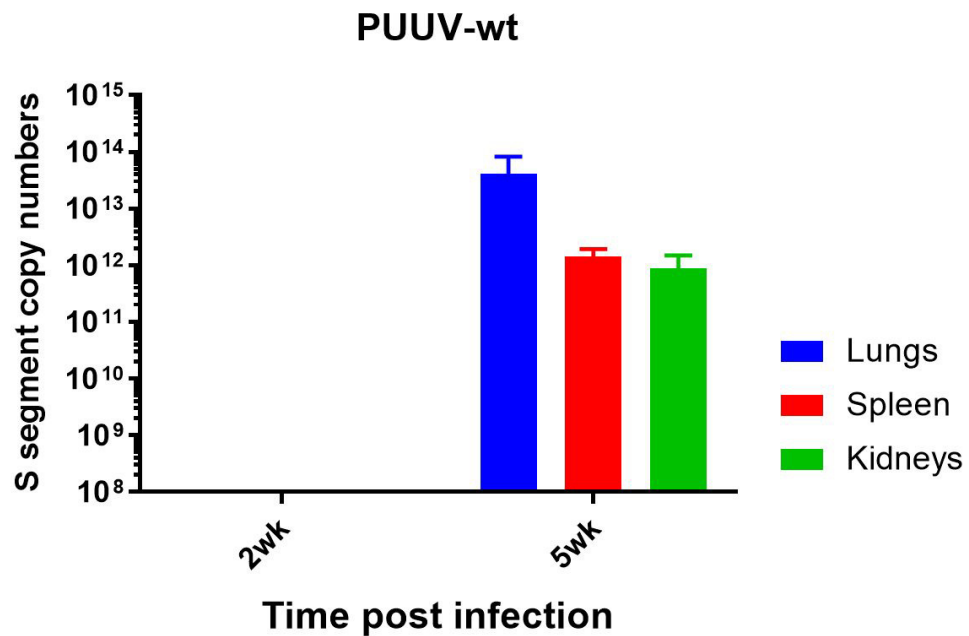
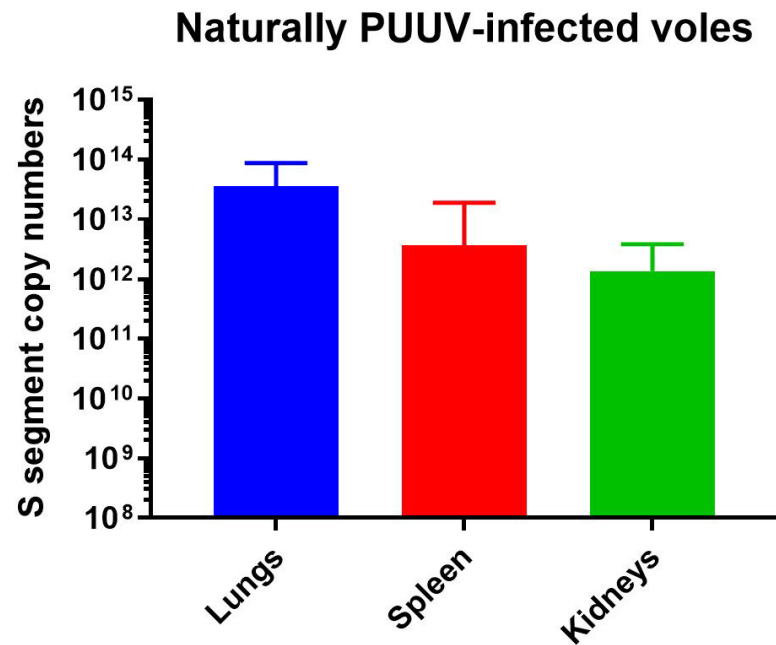
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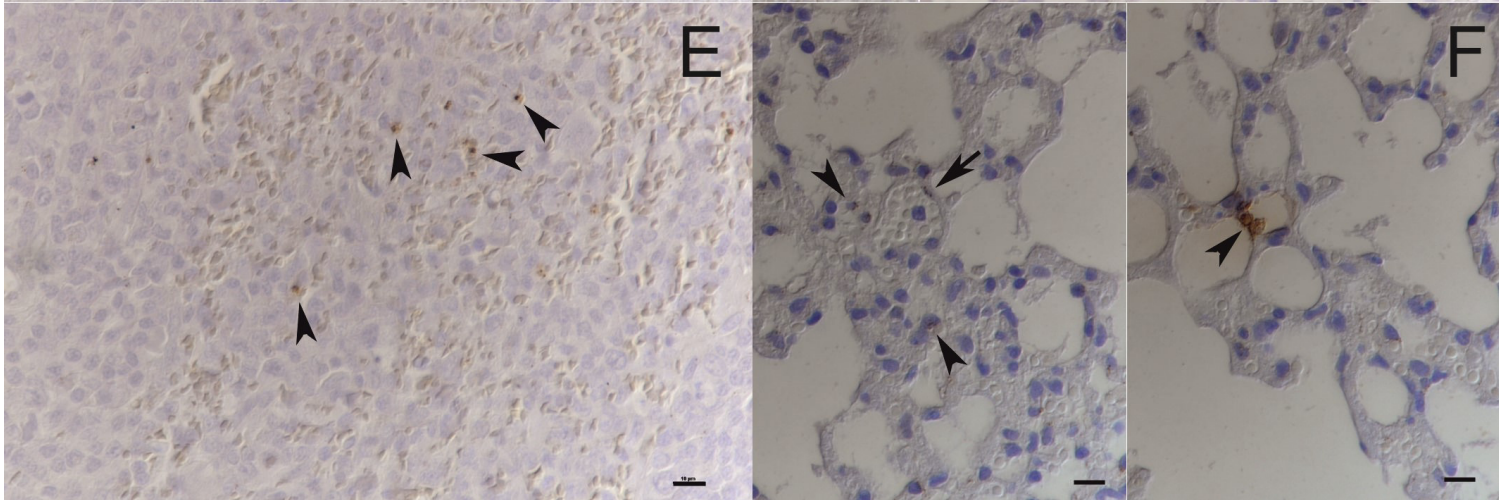
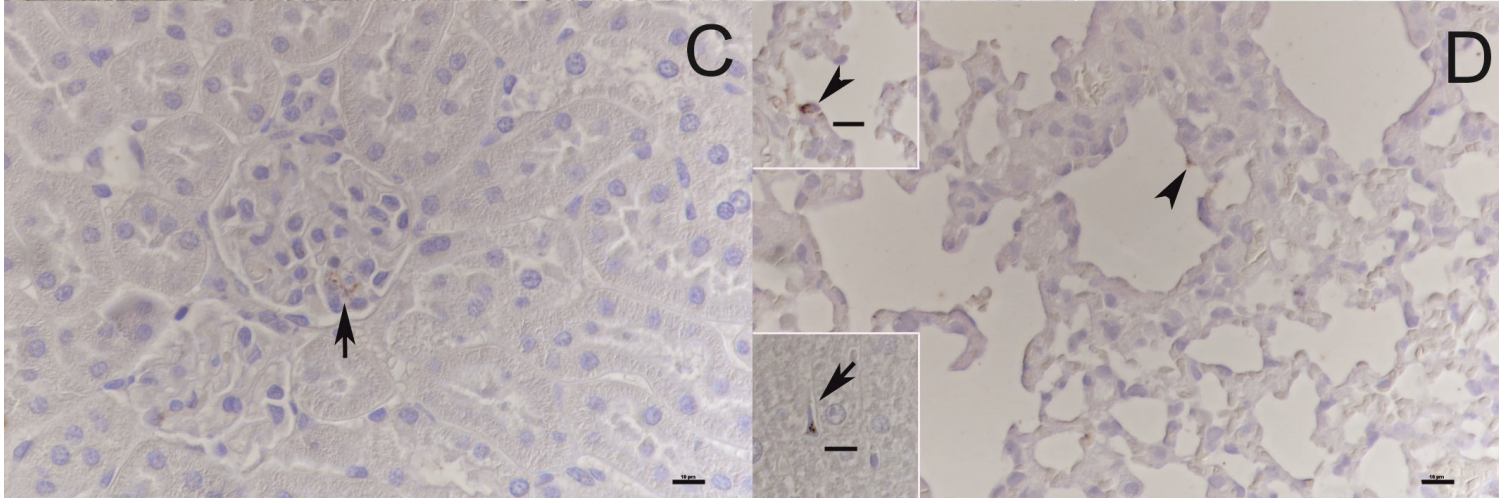
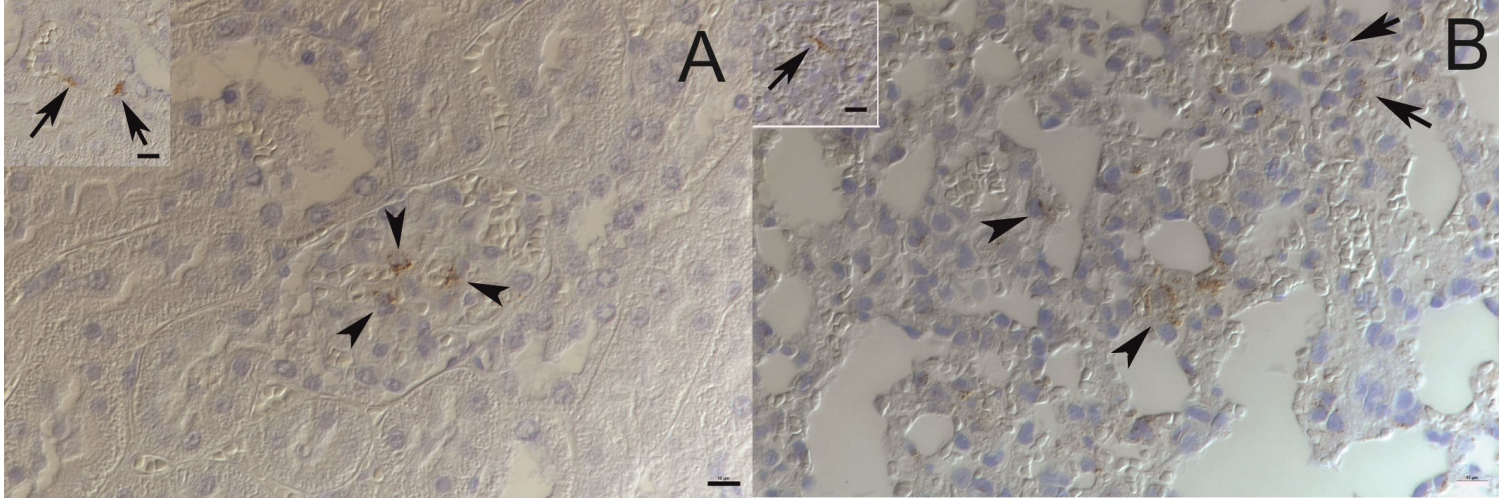


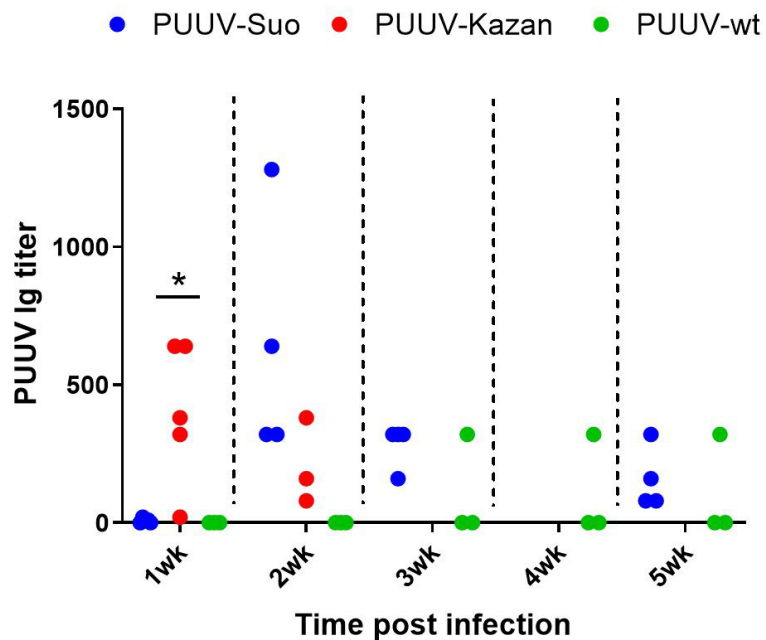
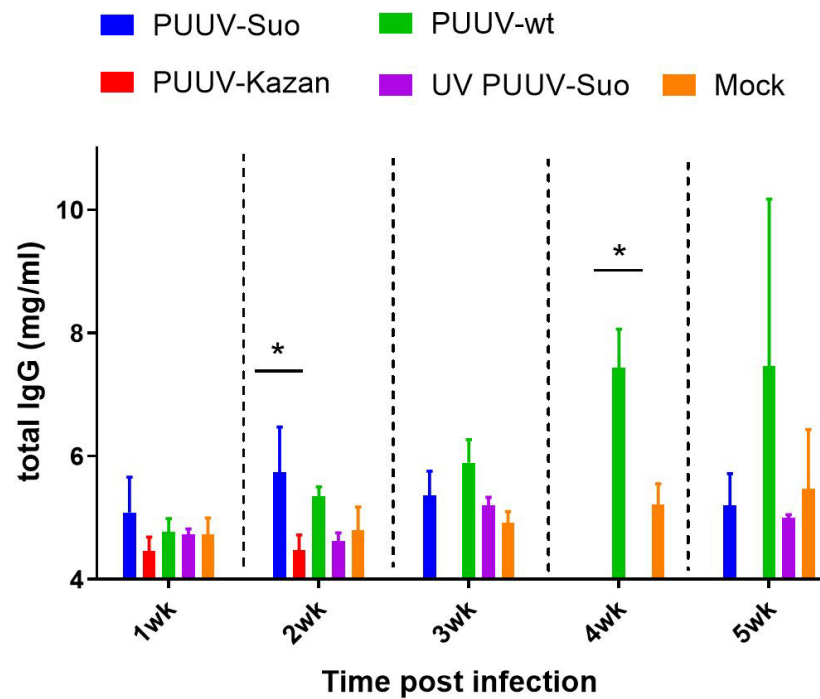
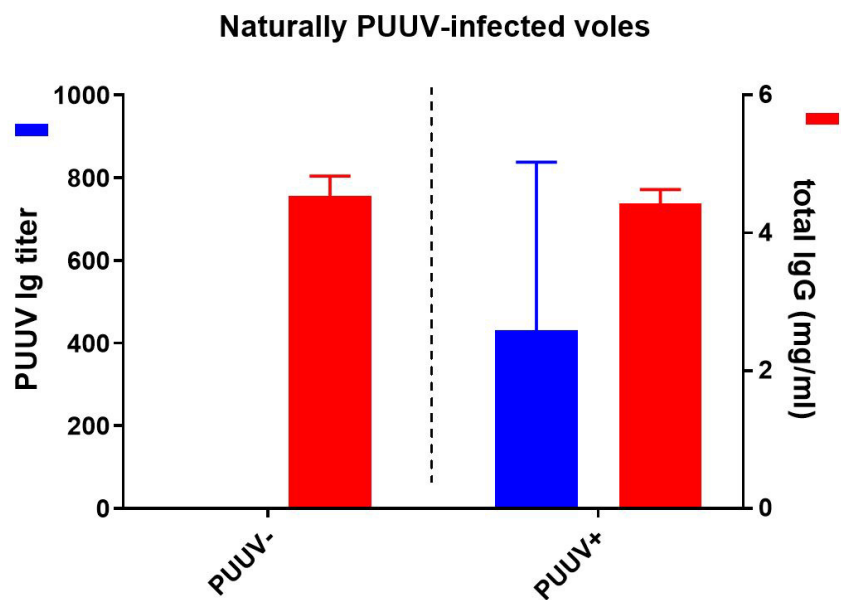
PUUV-Suo



A**B**

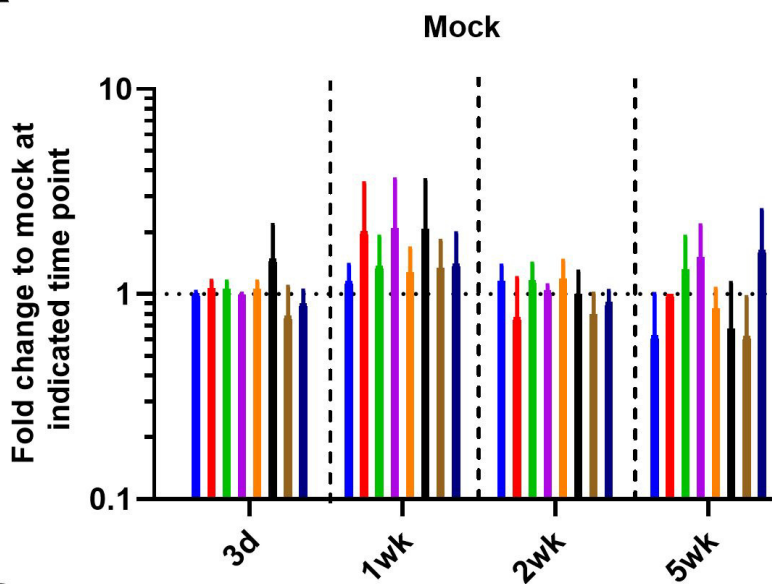
A**B****C****D**



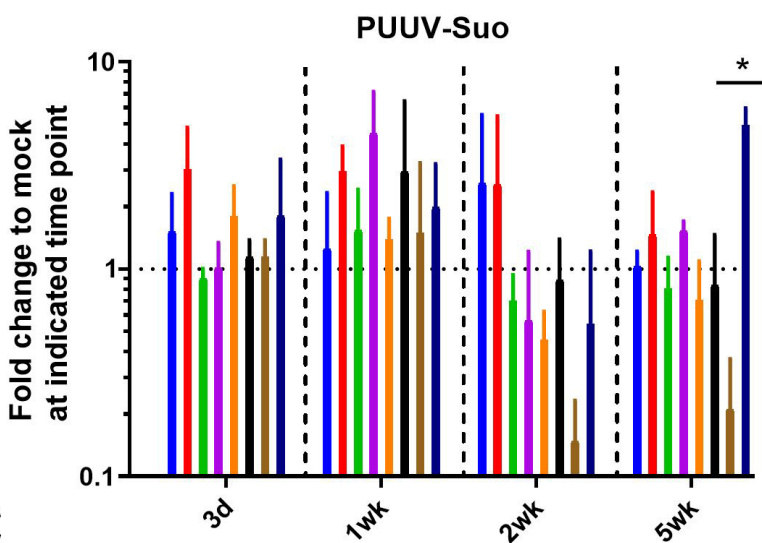
A**B****C**



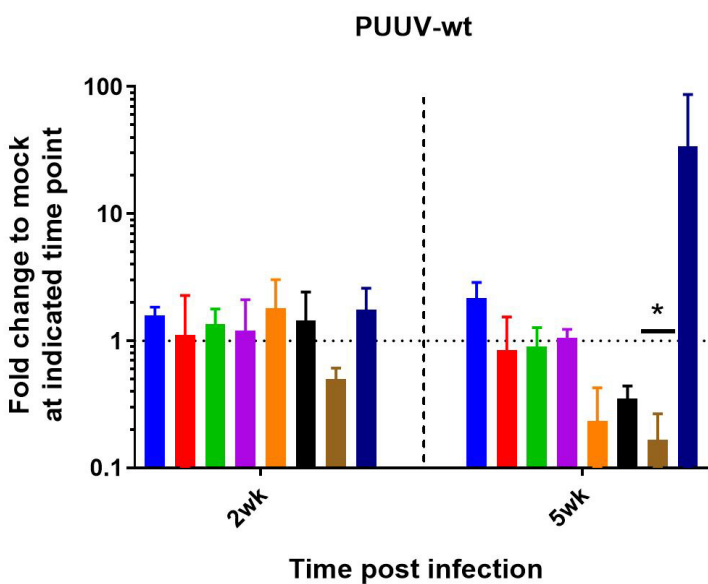
A



B



C



Fold change mRNA levels
to mock control

