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## Dengue virus NS1 protein conveys pro-inflammatory signals by docking onto high-density lipoproteins

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2 **Title: Dengue virus NS1 protein conveys pro-inflammatory signals by**  
3 **docking onto high-density lipoproteins**

4  
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49

50 **Short title:** NS1 forms a pro-inflammatory complex with HDL

51 **Key words:** Arbovirus, hemorrhagic fever, virulence factor, accessory protein, lipoprotein  
52 particle, molecular pathogenesis

53 **ABSTRACT**

54 The dengue virus nonstructural protein 1 (NS1) is a secreted virulence factor that modulates  
55 complement, activates immune cells and alters endothelial barriers. The molecular basis of  
56 these events remains incompletely understood. Here we describe a functional high affinity  
57 complex formed between NS1 and human high-density lipoproteins (HDL). Collapse of the  
58 soluble NS1 hexamer upon binding to the lipoprotein particle leads to the anchoring of  
59 amphipathic NS1 dimers into the HDL outer layer. The stable complex can be visualized by  
60 electron microscopy as a spherical HDL with rod-shaped NS1 dimers protruding from the  
61 surface. We further show that the assembly of NS1-HDL complexes triggers the production  
62 of pro-inflammatory cytokines in human primary macrophages while NS1 or HDL alone do  
63 not. Finally, we detect NS1 in complex with HDL and low-density lipoprotein (LDL) particles  
64 in the plasma of hospitalized dengue patients and observe NS1-apolipoprotein E-positive  
65 complexes accumulating overtime. The functional reprogramming of endogenous lipoprotein  
66 particles by NS1 as a means to exacerbate systemic inflammation during viral infection  
67 provides a new paradigm in dengue pathogenesis.

68

## 69 INTRODUCTION

70 Dengue virus (DENV) infects nearly 400 million people annually, leading to more than  
71 500,000 hospitalizations (Bhatt *et al*, 2013; Wilder-Smith *et al*, 2019). The mortality rate  
72 varies from less than 1% to 10% depending on the epidemic and medical care provided to  
73 patients (« Dengue and Severe Dengue » s. d. WHO, 2016; (Yacoub *et al*, 2016)). The dengue  
74 nonstructural protein 1 (NS1) is a viral effector circulating in the bloodstream of DENV-  
75 infected patients (reviewed in (Glasner *et al*, 2018; Rastogi *et al*, 2016; Watterson *et al*,  
76 2016)). In DENV-infected cells, NS1 forms amphipathic dimers in the endoplasmic reticulum  
77 (ER) that insert into the luminal side of the membrane (Akey *et al*, 2015; Lindenbach & Rice,  
78 1997; Winkler *et al*, 1989). The membrane-bound dimers play an essential role in  
79 orchestrating viral replication in specialized subcellular factories (Lindenbach & Rice, 2003).  
80 A sub-fraction of NS1 dimers further associate by three to form barrel-shaped hexamers.  
81 During this process, NS1 hexamers detach from the ER membrane and are secreted as soluble  
82 nanoparticles loaded with lipids from the infected cell (Flamand *et al*, 1999; Gutsche *et al*,  
83 2011). The secreted form of NS1 has previously been shown to bind to complement and  
84 coagulation factors, to activate immune and endothelial cells, to trigger the expression of pro-  
85 inflammatory cytokines, to alter the glycocalyx barrier and to promote endothelium  
86 permeability (Beatty *et al*, 2015; Flamand *et al*, 2009; Modhiran *et al*, 2017; Modhiran *et al*,  
87 2015; Puerta-Guardo *et al*, 2019; Puerta-Guardo *et al*, 2016). The antibody response against  
88 NS1 has been shown to protect against several flavivirus infections (Beatty *et al*, 2015; Brault  
89 *et al*, 2017; Espinosa *et al*, 2019; Schlesinger *et al*, 1985, 1987) but can also be harmful *via* a  
90 cross-reaction with platelets and endothelial cell surface antigens (Falconar, 2007; Jayathilaka  
91 *et al*, 2018; Lin *et al*, 2006; Lin *et al*, 2011; Sun *et al*, 2007; Wan *et al*, 2016). These  
92 characteristics altogether favor the development of thrombocytopenia, vascular leakage and  
93 hemorrhage. Given the growing evidence of NS1 involvement in dengue pathogenesis, a

94 better understanding of the molecular fate of NS1 in extracellular fluids by identifying its  
95 interacting partners is of utmost importance.

96  
97 In the present study, we report that NS1 from dengue virus serotype 2 (DENV-2) binds high-  
98 density lipoproteins (HDL) and with a lower affinity low-density lipoproteins (LDL).  
99 HDL and LDL are lipoprotein complexes composed of large lipid bundles surrounded by the  
100 apolipoproteins A-I and B, respectively, as well as a panel of functional proteins recently  
101 identified by proteomic approaches (Birner-Gruenberger *et al.*, 2014; Ronsein & Vaisar,  
102 2019). Lipoprotein particles that circulate in the blood have long been recognized for their  
103 regulatory functions in vascular homeostasis, inflammation and innate immune responses  
104 (Birner-Gruenberger *et al.*, 2014; Camont *et al.*, 2011; Feingold & Grunfeld, 2000;  
105 Ramasamy, 2014; Saemann *et al.*, 2010). We explored the NS1-HDL association by  
106 biophysical methods and visualized the complex by electron microscopy, which revealed NS1  
107 dimers protruding on the HDL surface. We observed that the NS1-HDL complex could trigger  
108 the production of pro-inflammatory cytokines in primary human macrophages. In addition,  
109 we consistently detected elevated levels of NS1-HDL complexes in the blood of DENV-  
110 infected patients on the day of hospital admission using an anti-apolipoprotein A-I (ApoA-I)  
111 detection assay. NS1 complexes acquired an apolipoprotein E (ApoE)-positive phenotype  
112 during the clinical phase, a component mostly found on very-low-density lipoproteins or  
113 chylomicrons and only transiently associated with HDL or LDL in physiological conditions.  
114 This points to a complex and dynamic interaction of DENV NS1 with the host lipoprotein  
115 metabolic cycle.

116

## 117 **RESULTS AND DISCUSSION**

### 118 **The DENV-2 NS1 hexamer binds high- and low-density lipoprotein particles**

119 In this study, we first sought to identify NS1 protein partners encountered during its  
120 circulation in human blood. For this purpose, we carried out a pull-down assay using a  
121 purified preparation of recombinant streptavidin-tagged DENV2 NS1 spiked in plasma  
122 obtained from healthy donors to identify potential ligands. We then re-affinity purified NS1  
123 from the plasma and analyzed the resulting products by size exclusion chromatography (SEC)  
124 (Fig. 1A). Compared to NS1 alone, the pull-down SEC profile showed an additional peak and  
125 a large shoulder at smaller elution volumes, corresponding to apparent molecular weights of  
126 840 and 380 kDa, respectively (Fig. 1A). The protein content of the two high molecular  
127 weight complexes was analyzed by SDS-PAGE, and the identities of the predominant protein  
128 bands were determined by N-terminal sequencing and mass spectrometry as ApoA-I and  
129 ApoB. These proteins correspond to the main scaffold proteins of HDL and LDL, respectively  
130 (Fig. 1A). NS1-HDL and NS1-LDL complexes could also be detected in the extracellular  
131 media of S2 cells expressing recombinant NS1 and cultured in the presence of fetal bovine  
132 serum (FIG. EV1), as analyzed by size exclusion chromatography (Fig. EV1A), SDS-PAGE  
133 (Fig. EV1B) and negative stain electron microscopy (Fig. EV1C). As a matter of fact, in these  
134 experimental conditions NS1-ApoA-I and NS1-ApoB lipoprotein complexes were the  
135 predominant NS1 species and no significant level of free NS1 could be observed in the culture  
136 media (Fig. EV1A). Moreover, an association of native NS1 with a conformationally relevant  
137 ApoA-I could further be demonstrated by co-immunoprecipitation of DENV-infected cell  
138 culture supernatants supplemented with human serum using an anti-NS1 monoclonal  
139 antibody (MAb) or anti-ApoA-I polyclonal antibodies (PAb) (Fig. EV2A,B).

140



141 These observations prompted us to assess the affinity of DENV2 NS1 for HDL and LDL.  
142 We immobilized human HDL and LDL particles on bio-layer interferometry (BLI) sensors  
143 coated with specific polyclonal antibodies against ApoA-I or ApoB, respectively. Figure 1B  
144 displays the binding curves for increasing NS1 concentrations in contact with both types of  
145 lipoprotein particles and the values reached at steady state (also see Fig. EV3). The curves  
146 could be fitted using a single-state binding model, leading to relative binding constants ( $K_d$ )  
147 of  $63 \text{ nM} \pm 0.2 \text{ nM}$  and  $1.4 \text{ } \mu\text{M} \pm 0.1 \text{ } \mu\text{M}$  for HDL and LDL, respectively (Fig. 1B).

148  
149 In order to characterize the architecture of the complex, we used analytical ultracentrifugation  
150 to study the behavior of NS1, HDL and a mix of HDL and NS1 at a HDL:hexameric NS1  
151 molar ratio of 1:1 or 1:5 (Fig. 1C). Purified NS1 sedimented as a main species with a  
152 sedimentation coefficient of 7.9 S compatible with a globular hexamer. HDL particles  
153 exhibited a much lower value of 4.20 S in keeping with the larger lipid to protein ratio (Lauer  
154 *et al*, 2016). In the sample containing a 1:5 excess mass of NS1 relative to HDL, all the HDL  
155 was engaged in an interaction with NS1. The unique species that was formed sedimented with  
156 a coefficient of 16.8 S, segregating distinctly from the other species (Fig. 1C). Residual  
157 unbound NS1 hexamer could still be observed, as expected due to the NS1 excess (Fig. 1C).  
158 By combining two detectors and taking into account the theoretical composition of the HDL  
159 particles, we estimated that one NS1 hexamer was bound to each HDL particle when present  
160 in excess. Interestingly, in the 1:1 ratio sample, we detected intermediate peaks at 9.4 and  
161 11.7S that corresponded to one or two NS1 dimeric subunits bound to HDL, respectively.

162  
163 **The NS1 hexamer dissociates into discrete dimeric blocks on the surface of spherical**  
164 **HDL particles**

165 Based on the above results, we prepared NS1-HDL complexes at a 1.5:1 NS1 to HDL molar  
166 ratio and examined the resulting products by negative-stain electron microscopy (EM).  
167 As previously shown, human HDL particles appeared as smooth spheres  $\approx$ 10 nm in diameter  
168 with an electron-dense central region (Zhang *et al*, 2013) (Fig. 2A). The purified NS1-HDL  
169 complexes, in contrast, presented a granular surface with prominent structures on their outer  
170 layer (Fig. 2B). 2D class averages of NS1-HDL complexes revealed that the HDL particles  
171 were crowned with high-density features that match very well with the contour of NS1 dimers  
172 in side view with two discrete nodules that likely correspond to the NS1 protomers  
173 (Fig. 2B, 2C) (Fig. EV4A,B) (Akey *et al.*, 2015; Lindenbach & Rice, 1997; Winkler *et al.*,  
174 1989). Anti-NS1 Fabs confirmed the presence of NS1 dimers on the surface of HDL particles  
175 by forming salient outward projections (Fig. EV4C). Our analysis also revealed that  
176 about 60% of the NS1-HDL complexes presented three NS1 dimers on the HDL surface while  
177 around 25% and 10% of the particles presented 2 or 4 apparent NS1 dimers on their surface,  
178 respectively (Fig. 2B). These observations corroborated the ultracentrifugation data showing  
179 different ratios of NS1 dimers per HDL particle depending on the initial NS1:HDL ratio  
180 (Fig. 1C). This points to a dynamic binding mode between NS1 and HDL particles with, in  
181 particular, the collapse of NS1 hexamers into dimers upon binding to HDL particles as  
182 depicted in Fig. 2C.

183  
184 The presence of NS1 dimeric subunits associated with HDL could also be evidenced by  
185 differential scanning calorimetry (DSC) (Fig. 2D). Thermal scanning of both hexameric NS1  
186 and the NS1:HDL mixture (at a 1.5:1 molar ratio) showed two transition phases while the  
187 HDL particles alone did not contribute to any signal in the scanned temperature range  
188 (Fig. 2D) (Jayaraman *et al*, 2015). The second transition at a  $T_m$  of 81°C was identical for  
189 NS1 alone or in complex with HDL. We have previously reported that the NS1 dimer of

190 Japanese encephalitis virus requires temperatures higher than 80°C to dissociate into  
191 monomers (Flamand *et al.*, 1995). We therefore attributed this second transition to the  
192 dissociation and full denaturation of NS1 dimeric subunits (Fig. 2D). Accordingly, the first  
193 transition peak corresponds to the dissociation of NS1 hexamers into dimers for NS1 alone  
194 (T<sub>m</sub> of 59°C) and to the release of NS1 dimers from the HDL particle for the NS1-HDL  
195 mixture (T<sub>m</sub> of 67°C) (Fig. 2D). The difference in T<sub>m</sub> values observed for the first transition  
196 peak in both samples provides additional evidence that once the NS1-HDL complex formed,  
197 the NS1 dimer-dimer interface initially present in the hexamer is converted into a more stable  
198 interface formed between the NS1 dimers and the HDL surface.

199  
200 We showed in a previous study that the NS1 dimeric building blocks behave as hydrophobic  
201 entities in a Triton X-114 phase partitioning assay (Gutsche *et al.*, 2011). Others reported that  
202 NS1 has the ability to interact with cellular membranes and liposomes (Akey *et al.*, 2015;  
203 Jacobs *et al.*, 2000; Lindenbach & Rice, 1997; Winkler *et al.*, 1989). Thus, dimers have a  
204 propensity to interact with hydrophobic surfaces and lipids. The recent demonstration that  
205 NS1 binds ApoA-I through hydrophobic interactions (Coelho *et al.*, 2021) suggests that this  
206 interaction may be important in promoting the collapse of the NS1 hexamer into dimers on  
207 the surface of the HDL particle. We were also able to show that the interaction between NS1  
208 and HDL could be inhibited with anti-ApoA-I PAbs raising the question of the nature of the  
209 interaction between the NS1 hexamer and the ApoA-I protein at the initial stage of NS1  
210 binding to HDL (Fig. 2E).

211  
212 **The NS1-HDL complex triggers pro-inflammatory signals in human primary**  
213 **macrophages**

214 NS1 is known to trigger the production of pro-inflammatory cytokines in macrophages  
215 (Modhiran *et al.*, 2017). As NS1 associates to HDL, themselves potent modulators of  
216 inflammation (Camont *et al.*, 2011; Saemann *et al.*, 2010), we questioned the role of NS1  
217 versus its complex NS1-HDL form in this process and characterized the cytokine and  
218 chemokine production pattern in human macrophages exposed to NS1 alone, HDL alone or  
219 to the NS1-HDL complex (Fig. 3). Primary macrophages were differentiated from isolated  
220 monocytes of various donors and stimulated with the different combinations of effectors.  
221 When exposed to NS1 or HDL alone, we observed no significant difference in the cytokine  
222 levels produced by macrophages compared to the negative control, whereas the bacterial  
223 lipopolysaccharide (LPS) consistently induced high levels of cytokine secretion (Fig. 3A-D).  
224 These observations ruled out any cytotoxic effect from putative contaminants in the NS1 and  
225 HDL samples. In contrast, the NS1-HDL mixture induced an increase in TNF- $\alpha$  (Fig. 3A),  
226 IL-6 (Fig. 3B), IL-1 $\beta$  (Fig. 3C) and IL-10 (Fig. 3D) secretion compared to NS1 alone. The  
227 differences were higher and all significant when compared with HDL alone, suggesting that  
228 NS1 converts HDL into pro-inflammatory signaling particles (Fig. 3A-D).

229

### 230 **NS1-lipoprotein complexes are detected in hospitalized patients**

231 Knowing that the concentration of the above-mentioned cytokines and chemokines is  
232 dramatically increased in patients with severe dengue (Fink *et al.*, 2006; Green & Rothman,  
233 2006; Pang *et al.*, 2007; Yacoub *et al.*, 2013), we assessed the presence of NS1-HDL and NS1-  
234 LDL complexes in DENV-infected patients. To this end, we developed different ELISA  
235 formats to detect and quantify NS1-lipoprotein complexes in human plasma in addition to  
236 NS1 itself (Fig. EV5A-D). We tested blood samples from dengue patients on their days of  
237 admission and discharge from hospital (Fig. 4A-E). This represented on average a time  
238 interval of 4.3 days between the first and last samples. The vast majority of patients

239 (around 80%) showed significantly elevated NS1 and NS1-HDL, indicated by NS1-ApoA-I  
240 signals, in blood on the day of admission compared to the last sample (Fig. 4B,C). The highest  
241 concentrations were observed between day 2 and day 4 post-onset of fever and the signal  
242 waned to background levels by day 9. Over this period of time, levels of NS1-LDL complexes  
243 remained relatively low (Fig. 4E), despite the fact that 69% of the samples tested were  
244 positive. The size of the scaffold apolipoprotein B (ApoB, over 500 kDa) could not account  
245 for an accessibility issue of the protein to the detection antibodies, as opposed to ApoA-I of  
246 25 kDa in size. This difference likely resulted from a lower affinity of NS1 for LDL relative  
247 to HDL, as suggested by our *in vitro* observations (Fig. 1B), or to a lower concentration of  
248 LDL in DENV-infected individuals compared to HDL. Apolipoprotein E (ApoE), which  
249 associated to native NS1 in DENV-infected cell supernatants (Fig. EV2B), appeared as  
250 another marker of NS1-lipoprotein complexes. As opposed to the NS1-ApoA-I trend, NS1-  
251 ApoE concentrations increased over time and were the highest when tested on blood  
252 specimens recovered at the time of patient discharge from hospital (Fig. 4D). Interestingly,  
253 important changes in lipid concentrations could be observed over the same period of time  
254 with a transient drop in cholesterol and a concomitant rise in triglyceride (Fig. 4F-H). These  
255 findings raise the question as to whether the DENV NS1 protein accounts for these  
256 fluctuations and to which extent the viral protein impacts host lipid and lipoprotein metabolic  
257 pathways. This concern extends to other flaviviruses as well as we found that different flavivirus  
258 NS1 proteins have the ability to associate to HDL particles (Fig. 4I).

259  
260 Dengue virus NS1 is a viral virulence factor that contributes to the development of severe  
261 dengue, characterized by cytokine storm, thrombocytopenia, vascular leakage and  
262 hemorrhage (Akey *et al.*, 2015; Glasner *et al.*, 2018; Rastogi *et al.*, 2016; Watterson *et al.*,

263 2016). NS1 circulates in the blood of DENV-infected patients at nanogram to microgram per  
264 ml levels (Alcon-LePoder *et al*, 2006; Antunes *et al*, 2015; Libraty *et al*, 2002). NS1 can  
265 trigger the production of inflammatory cytokines and chemokines in cell culture and in  
266 immunodeficient mice (Alayli & Scholle, 2016; Beatty *et al.*, 2015; Chen *et al*, 2015;  
267 Modhiran *et al.*, 2015). Our findings demonstrate that the formation of NS1-HDL complexes  
268 is a prerequisite for this effector function. The association of NS1 and HDL triggers pro-  
269 inflammatory signals in primary human macrophages while NS1 or HDL alone have no  
270 comparable effect. Also, an interaction of NS1 with a lipid-free ApoA-I purified from  
271 inclusion bodies and refolded *in vitro* generates inactive complexes (Coelho *et al.*, 2021). It  
272 has long been recognized that mature HDL particles, in which the scaffold ApoA-I protein  
273 interacts with specific classes of lipids and accessory proteins (Gogonea, 2015), have an anti-  
274 inflammatory regulatory function and contribute to the maintenance of vascular integrity  
275 under physiological conditions (Birner-Gruenberger *et al.*, 2014; Camont *et al.*, 2011;  
276 Ramasamy, 2014; Saemann *et al.*, 2010). However, the recruitment of certain proteins by  
277 HDL, such as serum amyloid A (SAA), confers a pro-inflammatory status to these particles  
278 during an acute phase response (Kopecky *et al*, 2017; Marsche *et al*, 2013; Murch *et al*, 2007;  
279 Prufer *et al*, 2015; Wu *et al*, 2004). Our working hypothesis is that NS1 exerts a similar  
280 control on HDL during DENV infections. This process could involve the HDL scavenger  
281 receptor B1 that has recently been identified as a cell surface receptor for DENV NS1 (Alcala  
282 *et al*, 2022), allowing its internalization in many mammalian cell types including  
283 macrophages, endothelial cells, keratinocytes and hepatocytes. An NS1-HDL contribution to  
284 the cytokine storm would have a direct impact on the development of severe dengue, as  
285 increased levels of TNF $\alpha$ , IL6 and IL-10 correlate consistently with disease severity and in  
286 certain instances endothelium permeability (Abhishek *et al*, 2017; Dewi *et al*, 2004; Huang

287 *et al*, 2018; Lee *et al*, 2016; Rathakrishnan *et al*, 2012; Srikiatkachorn *et al*, 2017;  
288 Tramontini Gomes de Sousa Cardozo *et al*, 2017).

289  
290 Several studies have described altered levels of HDL, LDL or VLDL in severe dengue  
291 (Barrientos-Arenas *et al*, 2018; Biswas *et al*, 2015; Lima *et al*, 2019; Marin-Palma *et al*, 2019;  
292 Suvarna & Rane, 2009; van Gorp *et al*, 2002). It is conceivable that NS1 broadly impacts the  
293 lipoprotein network by modifying the signaling patterns associated to the different lipoprotein  
294 particles or modulating their metabolic turnover. In this respect, we report a dynamic  
295 interaction of NS1 with host lipoproteins illustrated by a predominant binding to ApoA-I and  
296 ApoB-positive lipoprotein complexes, representative of HDL and LDL species, and the  
297 acquisition of an ApoE-positive signature over the course of the disease. ApoE is an  
298 exchangeable lipoprotein that can associate with most lipoprotein particles during the lipid  
299 metabolic cycle (Marais, 2019; Su & Peng, 2020). ApoE has also been recognized for its anti-  
300 inflammatory, anti-oxidative, anti-thrombotic and endothelial repair related properties (Filou  
301 *et al*, 2016; Valanti *et al*, 2018). Further studies are now needed to investigate whether the  
302 formation of NS1-ApoE-positive lipoprotein complexes is part of a recovery mechanism from  
303 the host or whether NS1 continues its pathogenic reprogramming during the convalescent  
304 phase by interfering with ApoE function. A number of studies have described a persistence  
305 of asthenia for weeks in dengue patients, extending well beyond the end of the acute clinical  
306 phase (Halsey *et al*, 2014; Luengas *et al*, 2015; Teixeira *et al*, 2017; Tiga-Loza *et al*, 2020).

307  
308 We previously described that NS1 is secreted from DENV-infected cells as an atypical  
309 lipoprotein hexamer (Gutsche *et al*, 2011). Once bound to an HDL particle, the NS1 hexamer  
310 appears to collapse into its dimeric building blocks that eventually stick to the surface of the  
311 lipoprotein particle. It is not clear at this stage to which extent protein-lipid or protein-protein

312 interactions prevail but both are likely to be important. Indeed, NS1 dimers are known to have  
313 the ability to bind lipid membranes, liposomes and separate in detergent phases<sup>7-9</sup> and all the  
314 candidate NS1 protein ligands published so far belong to the HDL proteome (Avirutnan *et al.*,  
315 2006; Chung *et al.*, 2006; Coelho *et al.*, 2021; Conde *et al.*, 2016; Kurosu *et al.*, 2007; Lin *et*  
316 *al.*, 2012; Shao & Heinecke, 2018). These proteins include the scaffold protein ApoA-I,  
317 complement factors C4, C1s, hnRNP C1/C2, factor H, prothrombin, as well as inhibitory  
318 factors of complement clusterin, C5-9 and SC5b-9 complexes (Avirutnan *et al.*, 2006; Chung  
319 *et al.*, 2006; Coelho *et al.*, 2021; Conde *et al.*, 2016; Kurosu *et al.*, 2007; Lin *et al.*, 2012).  
320 The interaction between NS1 and ApoA-I is reported to involve hydrophobic interactions  
321 (Coelho *et al.*, 2021), suggesting that once the NS1 hexamer binds the HDL particle,  
322 an interaction between NS1 and ApoA-I could trigger the dissociation of the NS1 hexamer in  
323 favor of a more stable dimer-ApoA-I interface. This is corroborated by our finding that the  
324 NS1 dimeric subunits have a higher thermal requirement to dissociate from the HDL particle  
325 than from the NS1 hexamer itself (Fig. 2D). It should be noted though that the docking of  
326 NS1 to HDL particles may involve other binding determinants than ApoA-I, as NS1 also  
327 binds to LDL in which ApoA-I is poorly represented. Proteomic studies, direct protein-protein  
328 interaction assays or the use of synthetic lipoprotein particles with defined compositions will  
329 be instrumental in delineating the role of the different NS1 partners in the formation of  
330 biologically relevant NS1-lipoprotein complexes.

331  
332 In conclusion, we provide evidence of a direct binding of NS1 to the surface of spherical HDL  
333 particles and to a lesser extent LDL particles as well. Once bound to HDL, NS1 undergoes a  
334 structural transition that results in the dissociation of NS1 hexamers and the anchoring of its  
335 amphipathic dimeric subunits onto the HDL surface. The association of NS1 and HDL  
336 triggers pro-inflammatory signals in primary macrophages, as a possible means to increase



337 vascular permeability and virus propagation in the infected organism. We further show that  
338 NS1-HDL concentrations are the highest at the time patients are admitted at hospital and by  
339 the end of the hospitalization period, NS1 complexes acquire an ApoE-positive signature the  
340 function of which remains to be explored. Other biological questions that need to be addressed  
341 relate to the molecular determinants of NS1 binding to various types of lipoprotein species,  
342 the receptors engaged by NS1-lipoprotein complexes during host cell interactions,  
343 their respective contribution to signal transduction and the role of proteins and lipids in the  
344 overall dynamics of the system. Unraveling the molecular mechanisms governing the  
345 assembly of the NS1-HDL complex, its metabolic fate and pathogenic functions will be  
346 critical in defining preventive measures against dengue and possibly other flaviviruses of  
347 public health concern.

348

349 **MATERIALS AND METHODS**

350 **Cell line and viral infection**

351 Vero cells (ATCC CRL-1586) were grown at 37°C with 5% CO<sub>2</sub> in DMEM (Gibco)  
352 supplemented with 10% fetal calf serum (Gibco) and 1% penicillin/streptomycin. Vero cells  
353 were tested negative in mycoplasma with the MycoAlert Mycoplasma Detection kit (Lonza,  
354 LT07-318). Vero cells were infected with DENV type 2 (strain 16681) at a multiplicity of  
355 infection of 1 and incubated for 3 days at 37°C with 5% CO<sub>2</sub>.

356

357 **DENV-2 NS1 protein expression, purification and serum pull-down experiments**

358 The DENV-2 recombinant NS1 protein was expressed in *Drosophila* S2 cells and purified  
359 from the extracellular medium as detailed in the previously published supplementary methods  
360 (Gutsche *et al.*, 2011). Purified DENV-2 recombinant NS1 protein was incubated for 1h at  
361 37°C in serum or plasma recovered from a healthy donor (provided by the ICAReB facility,  
362 Institut Pasteur) at a final concentration of 400 µg NS1/mL plasma. The mix was then purified  
363 on a Strep-tactin column (Iba), washed twice with PBS Mg<sup>2+</sup>/Ca<sup>2+</sup> (Gibco) followed by 14  
364 column volumes of PBS 0.3 M NaCl and another 5 column volumes of PBS Mg<sup>2+</sup>/Ca<sup>2+</sup>.  
365 Elution was performed using 2.5 mM D-desthobiotine (Iba) in PBS Mg<sup>2+</sup>/Ca<sup>2+</sup>.

366 Purified samples of recombinant NS1, human HDL (Merck Millipore), human LDLs (Merck  
367 Millipore) or an *in vitro* reconstituted NS1-HDL mix were analyzed by size exclusion  
368 chromatography on a Superdex 200 10/300 column (GE healthcare). Protein standards from  
369 Bio-Rad were used to interpret elution profiles. The protein samples from the major peaks  
370 were further denatured in 5x Laemmli sample buffer containing β-mercaptoethanol, boiled  
371 for 5 min at 95°C and separated by discontinuous sodium dodecyl sulfate (SDS) 4-15%

372 polyacrylamide gel electrophoresis (SDS-PAGE precast gels, Bio-Rad). The SDS-PAGE gels  
373 were stained in Coomassie Blue solution (Bio-Rad).

374

### 375 **Biolayer Interferometry**

376 DENV2 NS1 binding to HDL and LDL particles was monitored by Biolayer Interferometry  
377 (BLI), using an Octet Red384 instrument (ForteBio). Streptavidin-coated biosensors (SA,  
378 ForteBio) were loaded with biotinylated anti-ApoA-I or anti Apo-B antibodies (Abcam),  
379 followed by HDL or LDL, respectively. Subsequently the biosensors were incubated in wells  
380 containing serial dilutions of NS1 protein (concentrations ranging from 6.25 to 800nM for  
381 HDL, and from 36 to 2500nM for LDL) and the BLI association signals were recorded in  
382 real-time until they reached a plateau. Finally, the biosensors were incubated in wells  
383 containing buffer to monitor the dissociation of the complexes formed, before being  
384 regenerated for further use in replicate experiments. The regeneration protocol, comprising  
385 three subsequent 20 seconds washes in 10 mM Gly-HCl pH2, could be applied up to eight  
386 times for up to two days without losing any loading capacity of the immobilized biotinylated  
387 antibodies. The specific NS1 binding curves were obtained by subtracting the non-specific  
388 signals measured on unloaded biosensors used as control references. The steady-state signals  
389 were determined at the end of the association step and fitted using the following equation:  
390  $Req = R_{max} * C / (Kd + C)$  where Req is the steady-state BLI response, C the NS1 concentration,  
391 and Rmax the response at infinite concentration. All measurements were performed at least  
392 three times to determine experimental error. All experiments were performed at 20°C in PBS  
393  $Mg^{2+}/Ca^{2+}$  (Gibco) supplemented with 0.1% milk to minimize nonspecific binding, using 96-  
394 well half-area plates (Greiner Bio6One) filled with 150 µl per well, and a shaking speed of

395 1000 rpm. Data was processed using the Scrubber (v2.0 BioLogic), BIAevaluation 4.0  
396 (Biacore) and Profit (Quantumsoft) softwares.

397 Binding inhibition of NS1 to HDL with anti-ApoA-I antibodies was assessed using  
398 streptavidin-coated SA sensors (ForteBio) coated for 900s with biotinylated anti-ApoA-I  
399 polyclonal antibodies (Abcam, 5 $\mu$ g/ml in PBS-milk). Sensors were then further loaded for  
400 900s with purified HDL (Cell Biolabs, 20 $\mu$ g/ml). Half of the HDL-loaded sensors were then  
401 saturated with anti-ApoA-I antibodies (5 $\mu$ g/ml for 900s), while the other half were just  
402 washed with buffer. Finally, all sensors were incubated into an NS1 solution (200nM) for  
403 1200s, and the levels of NS1 binding recorded as described above.

404

405

#### 406 **Analytical ultracentrifugation**

407 NS1, HDL, and NS1-HDL mixtures at different molar ratios were incubated 1h at 37°C and  
408 centrifuged at 32,000 rpm for the complexes in a XL-I and an Optima AUC (Beckman  
409 Coulter) analytical ultracentrifuge, at 20°C in a four-hole AN 50-Ti rotor equipped with 3-  
410 mm and 12-mm double-sector aluminum epoxy centrepieces.

411 Detection of the biomolecule concentration as a function of radial position and time was  
412 performed by absorbance measurements at 250 nm and 280 nm and by interference detection.

413 Ultracentrifugation experiments were performed in PBS<sup>+/+</sup> (Gibco). Extinction coefficients  
414 were extrapolated at 250 nm using the Utrascan II software. Sedimentation velocity data  
415 analysis was performed by continuous size distribution analysis c(s) using the Sedfit 15.0  
416 software (Brown & Schuck, 2006). All the c(s) distributions were calculated with a fitted  
417 fractional ratio  $f/f_0$  and a maximum entropy regularization procedure with a confidence level  
418 of 0.95. Buffer viscosity and density as well as the extinction coefficient of NS1 were  
419 calculated using the sednterp software (<http://www.jphilo.mailway.com/download.htm>).

420 Molecular Mass and partial specific volume of NS1 and HDL were calculated from  
421 multidetection AUC experiment. NS1 have estimated mass for the monomer of 43 kDa with  
422 a partial specific volume of 0.721. HDL have estimated mass of 164 kDa, in agreement with  
423 mass photometry measurement (162 kDa, Fig. EV5F), and a partial specific volume of  
424 0.843 ml.g<sup>-1</sup> in agreement with the estimation from CsCl gradient (0.850 ml.g<sup>-1</sup>).  
425 Deconvolution of the multi-detector signal into stoichiometric ratio was performed by  
426 integrating all the peaks on each detector to determine the contribution of each partner present  
427 (NS1, HDL or both) and solving the contribution of each partner to the signal.  
428 Taking these measurements into consideration, we can convert a mass ratio to a molar ratio  
429 as follows: a NS1:HDL mass ratio of 1:1 corresponds to a 1:1.6 molar ratio.

430  
431 **Differential scanning calorimetry (DSC)**  
432 Thermal unfolding of NS1 and of the NS1-HDL complex were followed using a VP-Capillary  
433 DSC instrument (Malvern MicroCal) in PBS buffer. The concentration of the NS1 hexamer  
434 was 0.2 mg/ml and was used at a 2:1 molar ratio to form the NS1-HDL complex. At least two  
435 DSC scans were recorded for each sample. Human HDL (Merck Millipore) was incubated  
436 with NS1 for 1 h at 37°C prior to the DSC experiments. Scan rate was 100°C/h with a filtering  
437 period of 2. Thermograms were analyzed with the Origin software provided by the  
438 manufacturer.

439  
440 **Electron microscopy and image analysis**  
441 Solutions of NS1 and NS1-HDL were spotted on glow-discharged carbon grids, contrasted  
442 with 2% uranyl acetate and imaged with a Tecnai F20 microscope (Thermo Fisher, USA) in  
443 low-dose conditions. Automated acquisitions were performed using EPU software (Thermo

444 Fisher, USA) and images were acquired using a Falcon II (Thermo Fisher, USA) direct  
445 detector.

446 HDL and NS1-HDL images were CTF-corrected (phase flip) and sorted using the XMIP  
447 software (Velazquez-Muriel *et al*, 2005). Corrected images were imported in Relion (Scheres,  
448 2012). The recommended strategy for particle picking was applied as follow: a manual  
449 selection of particles compatible with the HDL or NS1-HDL size was performed on a small  
450 number (about fifteen) of images. A 2D classification (40 classes) was performed, and  
451 five representative well-defined classes were selected as template for the automatic  
452 picking, leading to about 30,000 particles. A 2D classification (200 classes) was then  
453 performed. Classes obviously corresponding to artefacts were suppressed and a final run of  
454 2D classification (200 classes) was carried out.

455

#### 456 **Capture ELISA of NS1-lipoprotein complexes**

457 Microtitration plates were coated overnight with purified mouse anti-NS1 monoclonal  
458 antibody (MAb DEN-2 17A12). Wells were saturated and washed before serial dilutions of  
459 human sera spiked with purified DENV-2 NS1 or DENV1- or DENV-2-infected human sera  
460 were added to wells for 2 h at room temperature. Wells were washed again and incubated for  
461 1 h at 37°C with anti-ApoA-I (Novus Biologicals), ApoB or ApoE (Merck Chemicals LTD)  
462 polyclonal antibodies followed by a peroxidase-conjugated secondary antibody (Jackson  
463 ImmunoResearch Laboratories) detected with a 3,3', 5,5'-tetramethyl-benzidine solution  
464 (UltraTMB, ThermoFischer). Negative controls were measured when the reaction was carried  
465 out in the absence of antigen. Absorbance values were corrected by subtracting the mean  
466 value of the signal measured for the negative controls.

467

468 **Flavivirus NS1 proteins binding to HDL in human plasma**

469 Purified NS1 from different flaviviruses (yellow fever, YF; ZIKA; West Nile, WN; Japanese  
470 encephalitis, JE; The Native Antigen Company) were spiked for 1h30 at 37°C in normal  
471 human plasma and NS1-HDL complexes were further detected by the NS1-ApoA-I complex-  
472 specific ELISA (see Fig. EV5C). Flavivirus NS1-ApoA-I complexes were captured using an  
473 immobilized anti-dengue NS1 MAb cross-reactive for flavivirus NS1 protein. Bound ApoA-  
474 I was further detected using a specific goat polyclonal antibody (Novus Biologicals) followed  
475 by a species-specific peroxidase-labeled secondary antibody (Jackson ImmunoResearch  
476 Laboratories). The concentration values reported on the x-axis are given as an NS1 equivalent  
477 concentration.

478

479 **Macrophages immune activation assay**

480 Human monocytes were isolated from buffy coats and differentiated into macrophages in  
481 medium supplemented with human AB serum, as previously described (Allouch *et al*, 2013).  
482 Briefly, PBMCs were isolated from whole blood using a Ficoll gradient centrifugation  
483 (Eurobio). CD14<sup>+</sup> cells were purified by magnetic bead separation of PBMCs using CD14<sup>+</sup>  
484 human positive selection kit (StemCell) and plated 1x10<sup>6</sup> cells/mL on Teflon plates (Sarstedt)  
485 with 7 ml per plate in the following medium: RPMI-1640 (Gibco), 2 mM L-glutamine  
486 (LifeTechnologies), 1% penicillin-streptomycin (10,000 units penicillin and 10 mg  
487 streptomycin/mL; Life Tech), 10 mM Na Pyruvate (Life Tech), 10 mM HEPES (Life Tech),  
488 1% MEM vitamins (Life Tech), 1% NEAA (Life Tech), 50 uM beta-mercaptoethanol (Life  
489 Tech), and 15% human serum (ICAReB facility, Institut Pasteur). Monocytes were cultured  
490 in differentiating medium for 6-8 days, after which the macrophages were scraped off Teflon

491 plates and counted. After spinning, the cells were resuspended at  $1 \times 10^6$ /mL in the same  
492 medium but with 5% FBS instead of human serum.

493 Macrophages were plated at 0.5 million cells per mL in P24 plates (Corning) and left 2 h in  
494 the incubator for cell sedimentation. Aliquotes of serum-free media (Optipro, Gibco) were  
495 supplemented with NS1 (10  $\mu$ g/mL), HDL (2.5  $\mu$ g/mL), a NS1-HDL mix at the same  
496 respective quantities (2.5 NS1:1 HDL molar ratio), an equivalent volume of PBS as negative  
497 control, LPS (100 ng/mL) as positive control, and incubated for 1h at 37°C. Macrophages  
498 were then exposed to the different suspensions for 24h before collecting supernatants.  
499 Inflammatory mediators were detected in clarified cell supernatants using a hMagnetic  
500 Luminex Assay 5 Plex, R&D Systems, Bio-Techne Ltd run on the BioPlex 200 System xMAP  
501 (BioRad Laboratories Inc.) as per the manufacturer's specifications. The antibody bead kit  
502 was designed to quantify IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ . Standards were run with each plate at  
503 every assay to titrate the level of cytokines present. Statistical analyses were performed in  
504 Prism 6.0 (GraphPad Software Inc.) Data are shown as individual points and means  $\pm$  SD.  
505 Significant testing was performed using 2-way ANOVA.

506

### 507 **DENV-infected patient sera**

508 Patients presenting acute dengue-like symptoms – between June and October of 2011 and  
509 2012 – were enrolled at the Kampong Cham Referral Hospital, Cambodia. Inclusion criteria,  
510 following the WHO 1997 classification scheme, were children between 2 and 15 years old  
511 who had fever or history of fever at presentation and onset of at least two of the following  
512 symptoms within the previous 72 hours: headache, retro-orbital pain, muscle pain, joint pain,  
513 rash, or any bleeding signs. We performed a prospective, monocentric, cross-sectional study  
514 of hospitalized children with severe and non-severe dengue. The study was approved by the



515 Cambodian National Ethics Committee for Human Research (approval #087NECHR/2011).  
516 All patient enrollment and blood sampling occurred after obtaining written informed consent  
517 from the patient's parents or guardians. The first visit was conducted at hospital admission.  
518 The day of onset of symptoms was defined as day 0 of the illness. The last visit was performed  
519 at the time of discharge for patients who recovered entirely, or as a follow-up visit for patients  
520 still in the critical phase. A clinical and biological follow-up including abdominal/chest  
521 ultrasound recording was conducted at each visit. DENV infection of hospitalized patients  
522 was confirmed by NS1 antigen detection using NS1-capture ELISA (Alcon-LePoder *et al.*,  
523 2006; Antunes *et al.*, 2015; Libraty *et al.*, 2002) and/or RT-qPCR and/or virus isolation on  
524 *Aedes albopictus* C6/36 cells on the plasma sample obtained at admission (Andries *et al.*,  
525 2015). We observed that the NS1-capture ELISA set-up based on the NS1 capture with the  
526 4F7 MAb and the NS1 antigen detection with a peroxidase-labeled 8G6 MAb could  
527 efficiently detect the soluble NS1 hexamer or NS1 dimers associated to HDL but not the NS1  
528 protein interacting with ApoE.

529

### 530 **Biosafety**

531 Dengue virus-infected plasma samples were handled in a dedicated biosafety level (BSL)-3  
532 laboratory. The biosafety manual describes standard and specific operating procedures. It is  
533 elaborated with the support of our institution and adopted by all BSL3 users.

534

535 **DATA AVAILABILITY**

536 No large primary datasets have been generated and deposited.

537

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561

## 562 **DISCLOSURE AND COMPETING INTERESTS STATEMENT**

563 Dr. Philippe Buchy is a former Head of Virology at Institut Pasteur du Cambodge and is  
564 currently an employee of GSK Vaccines, Singapore.

565 Part of the work is patented (PCT/EP2020/07714).

566

## 567 **AUTHORS CONTRIBUTION**

568 SB, K-HP, MD, FC, FB, FR and MF conceived and designed the experiments. SB, K-HP,  
569 MD, JEV, CT, GP-A, BR, SB, PE, XZ, AM, MH, SP, SBB, FC, FB, MF performed the  
570 experiments. SB, K-HP, JEV, CT, SP and FC expressed and purified the NS1 protein and its  
571 complexes. SB, K-HP, MD, BR, SB and PE carried out the biophysical characterization of  
572 protein complexes. GP-A, XZ and FB collected and processed the EM data and built the  
573 model of the NS1-HDL complex. K-HP, CT, M-NU, MH and MF elaborated and developed  
574 the different quantification assays formats. K-HP, CT, AS, PB, VD, PD and MF recruited the  
575 dengue patient and tested the biological samples. SB, K-HP, MD, EH, FC, FB, FR and MF  
576 wrote the manuscript. All authors discussed the experiments and approved the manuscript.

577

## 578 **ETHICS STATEMENT**

579 The study on dengue virus-infected patients was approved by the Cambodian National Ethics  
580 Committee for Human Research (approval #087NECHR/2011). All patient inclusion and  
581 blood sampling occurred after obtaining written informed consent from the patient's parents  
582 or guardians.

583 Primary monocyte-derived macrophages were isolated from healthy donor blood obtained  
584 from the French blood bank (Etablissement Français du Sang) as part of a convention with  
585 the Institut Pasteur. In accordance with French law, written informed consent to use the cells  
586 for clinical research was obtained from each donor.

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826

827 **FIGURE LEGENDS**

828

829 **Fig. 1: DENV NS1 binds to human high-density and low-density lipoproteins**

830 (A) Size exclusion chromatography profile of NS1 pull-down experiments showing a clear  
831 shift after incubation in complete or heat-inactivated human serum (solid and dotted black  
832 lines, respectively) from the same healthy donor compared to the NS1 protein alone  
833 (blue line). NS1 protein interaction partners were identified by SDS-PAGE and N-terminal  
834 sequencing as the Apolipoprotein B scaffold of the low-density lipoproteins (LDL) in the first  
835 SEC elution peak, and the ApoA-I protein scaffold of the high-density lipoproteins (HDL) in  
836 the second elution peak.

837 (B) Biolayer interferometry (BLI) profiles corresponding to the binding of NS1 at various  
838 concentrations respective to human HDL (left panel) and human LDL (central panel)  
839 particles. The concentration-dependence of the steady-state signal corresponding to the  
840 binding of NS1 to HDL (black dots) and LDL (white dots) is shown on the right-hand side  
841 panel. The measurements were replicated at least three times using novel biosensors and  
842 samples. Data points and error bars correspond to the mean  $\pm$  SD.

843 (C) Typical sedimentation coefficient distribution of NS1 or human HDL alone or pre-  
844 incubated together (mixture of NS1 and HDL at a 1:1 or 5:1 mass ratio) monitored using an  
845 interferometric detector. Peaks were integrated for all the detectors (interference and  
846 absorbance at 280nm). The calculated stoichiometries are indicated for each peak. Solutions  
847 were equilibrated at 20°C for 2h before sedimentation velocity analysis.

848

849

850 **Fig. 2: Analysis of NS1-HDL complexes by electron microscopy reveals the presence of**  
851 **NS1 dimers on the surface of HDL particles**

852 (A, B) Electron microscopy observations from left to right: a representative image, followed  
853 by the three most representative classes of (A) purified HDL particles and (B) NS1-HDL  
854 complexes. White bar: 50 nm, Black bar: 20 nm.

855 (C) Fitting of the NS1 3D structure of the dimeric form into the most abundant class of NS1-  
856 HDL complexes, suggesting a collapse of the NS1 hexamer into hydrophobic dimeric blocks  
857 that float and anchor into the HDL lipid phase.

858 (D) Differential scanning calorimetry (DSC) of NS1 alone (blue line) or of an NS1-HDL  
859 mixture at a 2:1 molar ratio (orange). Of note, the HDL particles alone did not generate any  
860 signal in the temperature range tested.

861 (E) Binding inhibition of hexameric NS1 to HDL with anti-ApoA-I polyclonal antibodies  
862 (anti-ApoA-I Ab) measured by BLI.

863

864 **Fig. 3: The NS1-HDL lipoprotein complex triggers pro-inflammatory signals in human**  
865 **primary macrophages**

866 (A-D) Human primary macrophages were incubated for 24h with the different potential  
867 effectors (NS1, HDL, mix NS1-HDL) or with control suspensions (PBS buffer, LPS, mix  
868 LPS-HDL). LPS stimulation was used as a positive control in the presence or absence of HDL  
869 and provided values consistent between experiments. Phosphate buffer used in the SEC  
870 purification step was used as a negative control. Cell culture supernatants were clarified and  
871 tested with a Luminex assay to quantify the amount of (A) TNF- $\alpha$ , (B) Il-6, (C) Il-1 $\beta$  and (D)  
872 Il-10 released in the extracellular medium. Data reported on the graphs correspond to  
873 biological replicates of macrophages isolated from four blood donors (n=9 for TNF- $\alpha$ , Il-6

874 and Il-1 $\beta$ , n=5 for Il-10). Data represent mean +/- SEM. A Mann-Whitney test was used to  
875 assess the statistical significance of differences observed between mean cytokine levels in  
876 different cell culture supernatants. Not significant: ns, \* p < 0.05, \*\*\* p < 0.001.

877

878 **Fig. 4. Different biological and virological parameters measured in human plasma.**

879 (A-H) DENV-infected hospitalized patients from the Kampong Cham Referral Hospital,  
880 Cambodia, presented either dengue with warning signs or severe dengue. Two blood samples  
881 were recovered for each patient on the day of hospital admission and during a follow-up visit  
882 that occurred before discharge from the hospital (on average 4 days apart). (A) Number of  
883 patient samples tested over the hospitalization period for their levels of (B) NS1, (C) NS1-  
884 ApoA-I, (D) NS1-ApoE and (E) NS1-ApoB complexes in addition to (F) total cholesterol,  
885 (G) HDL-cholesterol and (H) triglycerides. NS1-ApoA-I and NS1-ApoB complexes are  
886 representative of NS1-HDL and NS1-LDL complex species, respectively, while the NS1-  
887 ApoE-positive complexes remain to be fully characterized. Errors bars indicate SEM.

888 (I) Purified NS1 from different flaviviruses (yellow fever, YF; ZIKA; West Nile, WN;  
889 Japanese encephalitis, JE; The Native Antigen Company) were spiked in normal human  
890 plasma and NS1-ApoA-I complexes further detected by ELISA. Data represent the mean  
891 values of two technical replicates.



892 **EXPANDED VIEW FIGURE LEGENDS**

893

894 **Fig. EV1: The secreted form of DENV-2 NS1 binds to bovine HDL and LDL.**

895 (A-C) The NS1 protein was expressed in *drosophila* S2 cells cultured either in InsectXpress  
896 protein-free medium (Lonza) supplemented with 5% complete fetal bovine serum (FBS) or  
897 without FBS. NS1 was purified on a Streptactin affinity column before analysis in a size  
898 exclusion column (superdex 200 16/60).

899 (A) Typical chromatograms obtained with purified secreted DENV-2 NS1 (blue line) and  
900 DENV-2 NS1 bovine complexes (red line) are shown.

901 (B) Denaturing SDS-PAGE analysis of 1 fraction every 4 mL from 38 to 75 mL elution  
902 volume. Molecular weights of protein standards (Std) are expressed in kDa. Proteins were  
903 detected by UV (Stain-free, Biorad). The three major protein bands identified by mass-  
904 spectrometry are bovine ApoB (>250 kDa), DENV-2 NS1 (~50 kDa), and bovine ApoA-I  
905 (~25 kDa).

906 (C) Negative-stain electron micrographs of purified hexameric NS1 alone (left panel) or  
907 purified as a complex with ApoA-I (NS1-HDL, central panel) or with ApoB (NS1-LDL, right  
908 panel). Bar: 100 nm. Data are derived from at least two independent experiments.

909

910 **Fig. EV2: Co-immunoprecipitation of native NS1 and apolipoproteins from DENV-**  
911 **infected cells cultured in media supplemented with bovine or human serum.**

912 (A-B) Vero cells infected with DENV (strain 16681) at an MOI of 1 or uninfected were  
913 cultured for 3 days in media supplemented with 10% FBS or 10% human serum. Supernatants  
914 were clarified by centrifugation and immunoprecipitated with (A) anti-NS1 MAb 17A12 or  
915 anti-ApoA-I PAbs or (B) with anti-NS1 MAb 17A12 or anti-ApoE PAbs. The resulting  
916 products were separated by SDS-PAGE on stain-free gels, submitted to UV light and

917 visualized in a gel imager (G-Box, Syngene) (A,B), or transferred onto a PVDF membrane  
918 treated with biotinylated anti-ApoE PAb and streptavidin-HRP (B). Data shown are  
919 representative of two technical replicates.

920  
921 **Fig. EV3: Dose-dependent binding of NS1 to HDL particles monitored by biolayer**  
922 **interferometry.**

923 (A-C) Biolayer interferometry (BLI) profiles corresponding to the binding of NS1 at various  
924 concentrations respective to human HDL particles. Data from NS1 binding to (A) HDL  
925 loaded biosensors, (B) antibody activated biosensors and (C) the corresponding subtraction.  
926 Data was colored according to the NS1 concentration as 800 nM in Magenta, 400 nM in  
927 violet, 200 nM in blue, 100 nM in sky blue, 50 nM in green, 25 nM in apple green, 12.5 nM  
928 in orange and 6.25 nM in red.

929  
930 **Fig. EV4: Electron microscopy analysis of NS1-HDL complexes.**

931 (A, B) Classes of NS1 and NS1-HDL purified species from negative-stain electron  
932 microscopy (EM) images. NS1 and NS1-HDL were purified as described in Material and  
933 Methods. The corresponding protein fractions were recovered from size exclusion  
934 chromatography and analyzed by negative-stain EM. Automated acquisitions were performed  
935 using EPU software and images were acquired using a Falcon II direct detector. Images were  
936 CTF-corrected (phase flip) and sorted using the XMIP software 84. Sizes of squares are  
937 reported at the bottom right of each panel.

938 (C) Representative electron microscopy image of negatively-stained NS1-HDL complexes  
939 bound to Fab 17A12. NS1-HDL complexes were formed at a 1:1 molar ratio, purified by SEC  
940 and further incubated with Fab 17A12 at a molar ratio of 3 Fab:1 NS1-HDL complex. Samples  
941 were spotted on glow discharged grids and contrasted with 2% uranyl acetate. Images were

942 acquired on a Tecnai F20 microscope operated at 200kV using EPU software (Thermo-Fisher,  
943 USA) on a Falcon II camera, under low dose conditions. Bar: 20 nm. Image representative of  
944 at least four different fields.

945

946 **Fig. EV5: Standard calibration curves.**

947 (A-E) Detection of the DENV NS1 protein and NS1-ApoA-I, NS1-ApoB or NS1-ApoE  
948 complexes by sandwich ELISA. (A) Detection of the purified DENV NS1 has been  
949 described previously <sup>11</sup>. (B) NS1-ApoA-I, NS1-ApoB or NS1-ApoE complexes were  
950 formed in normal plasma spiked with purified NS1 at a known concentration. Capture of  
951 the NS1-ApoA-I, NS1-ApoB or NS1-ApoE complexes were carried out using an anti-NS1  
952 monoclonal antibody (MAb17A12). The detection of immobilized complexes was  
953 performed with an anti-ApoA-I, anti-ApoB or anti-ApoE polyclonal antibody followed by  
954 a species-specific secondary antibody. The concentration of the NS1-ApoA-I, NS1-ApoB  
955 or NS1-ApoE complexes is reported on the basis of 100% NS1 bound to (C) HDL, (D)  
956 LDL or (E) ApoE-positive lipoprotein particles, respectively. Detection limits of the NS1,  
957 NS1-ApoA-I, NS1-ApoB or NS1-ApoE assays were set as twice the mean value of signals  
958 obtained with normal human plasma in the absence of NS1, which corresponded to 0.5,  
959 17, 5 and 15 ng of an equivalent NS1 concentration per milliliter, respectively.

960 (F) Determination of the HDL molecular weight by mass photometry. Purified HDL were  
961 diluted at 5 µg/mL and deposited on the coverslip. Measurements were performed  
962 according to the procedure described in Wu and Piszczek (*Eur Biophys J*, **2021**, vol.  
963 50: 403–409). The results show an average mass of 162kDa for the overall distribution.

964

965

Fig. 1

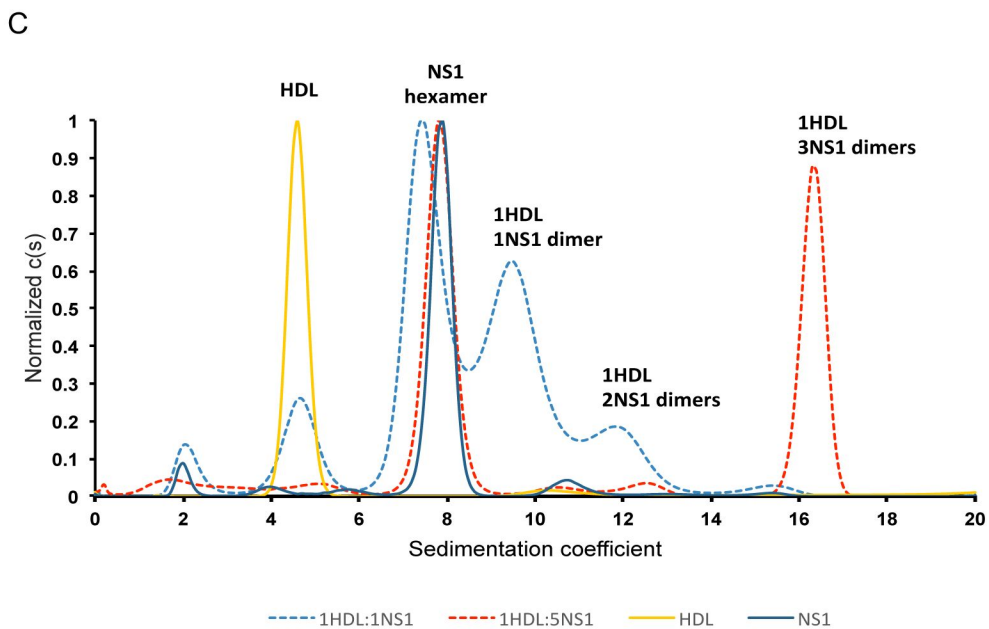
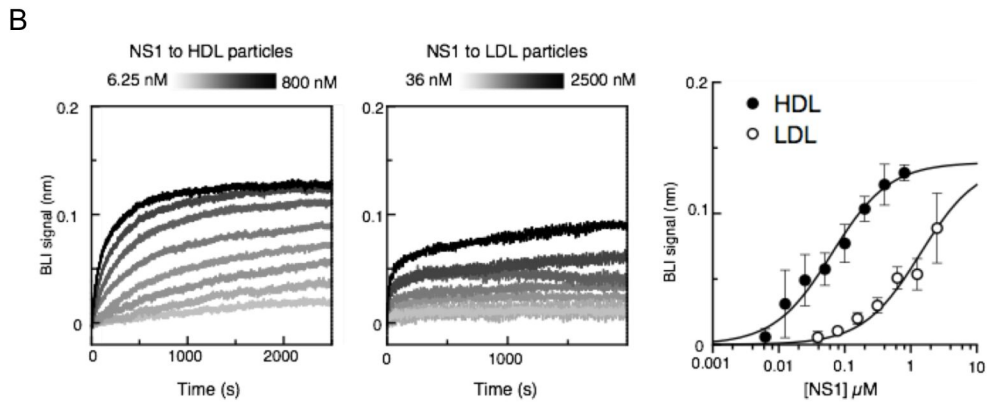
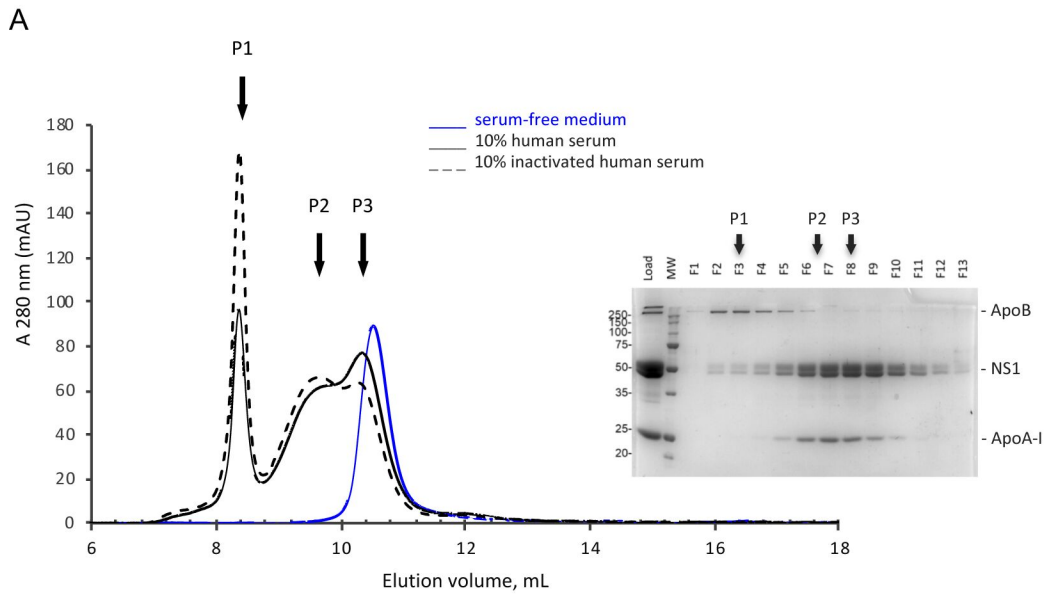


Fig. 2

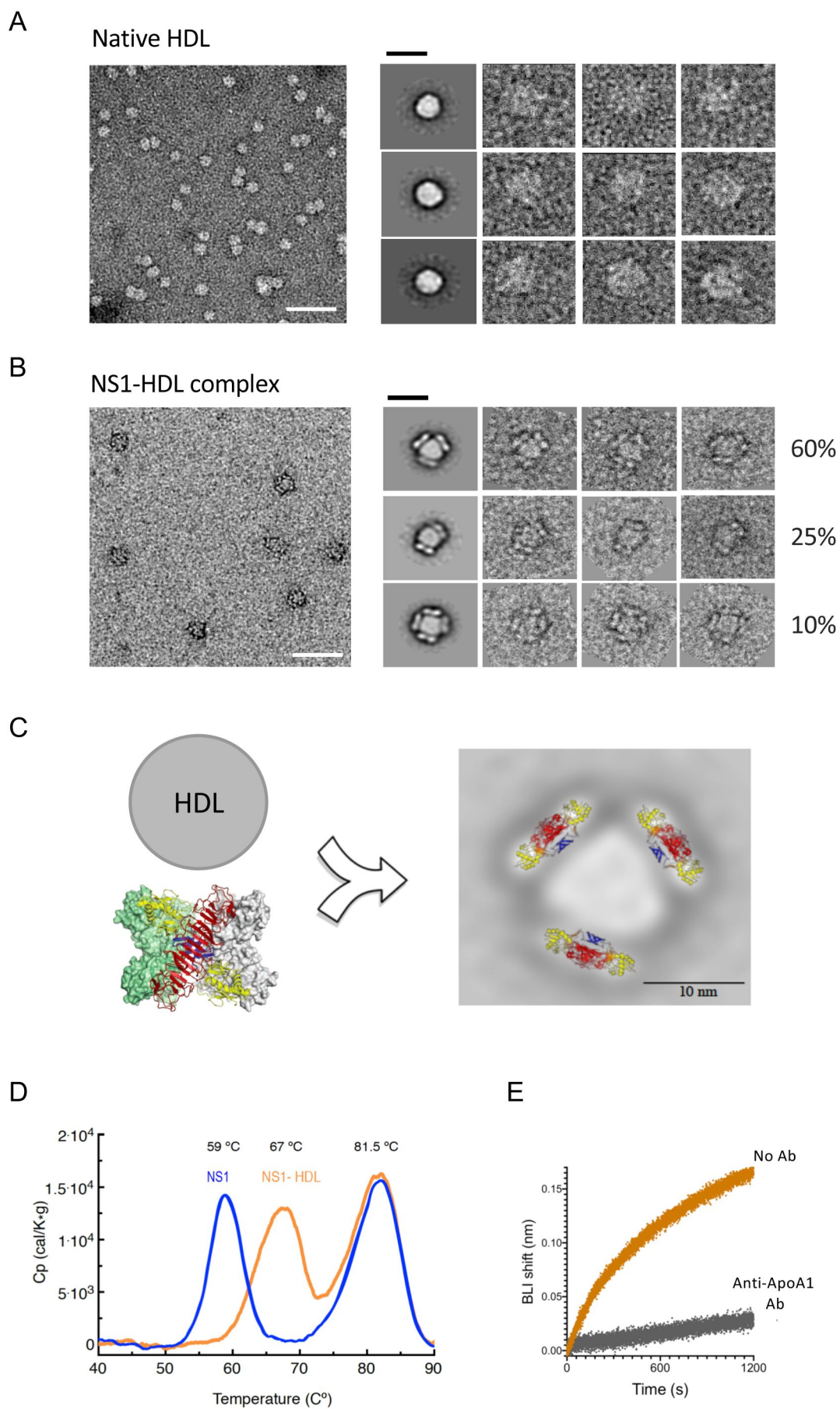
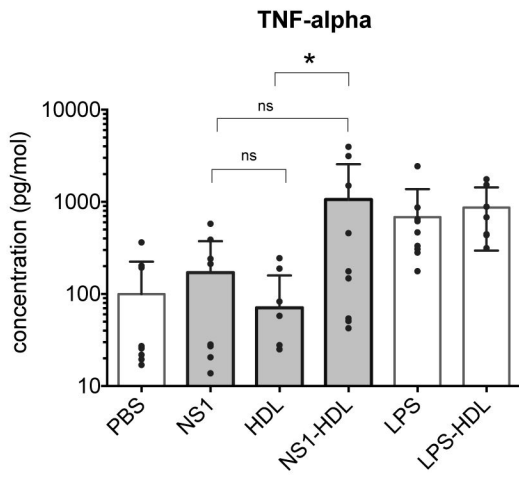
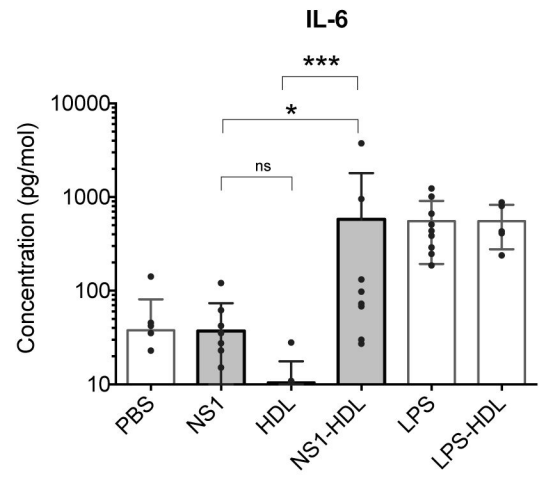


Fig. 3

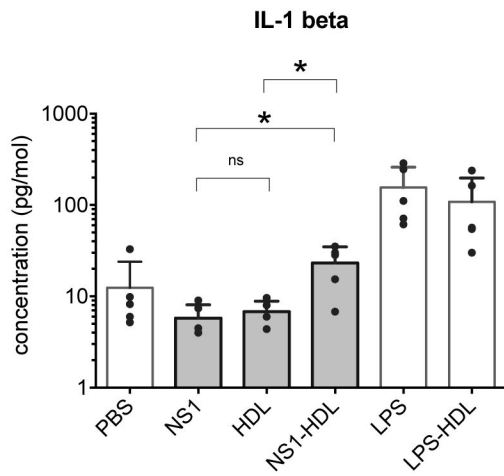
A



B



C



D

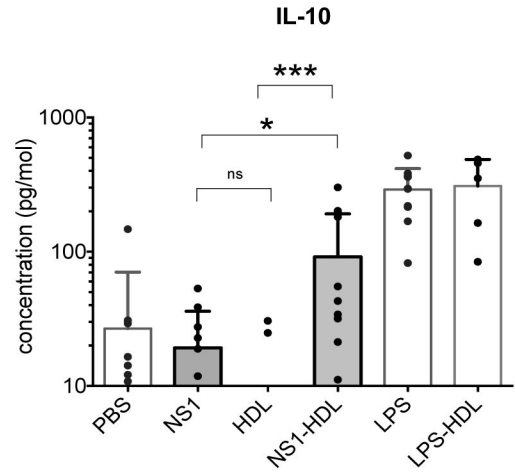
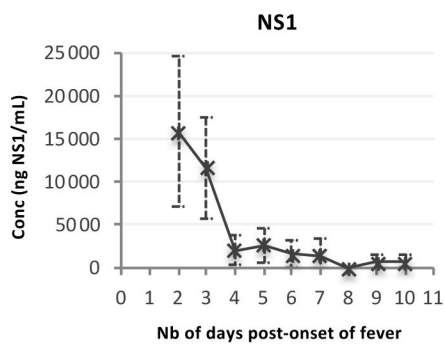


Fig. 4

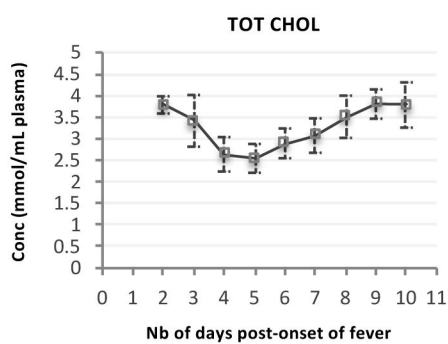
A

Nb of days post-onset of fever		2	3	4	5	6	7	8	9	10
N= nb of patient samples	Panel B-C-D Panel F-G-H	5	6	11	21	11	18	13	12	5
	Panel E	9	5	7	7	4	3			

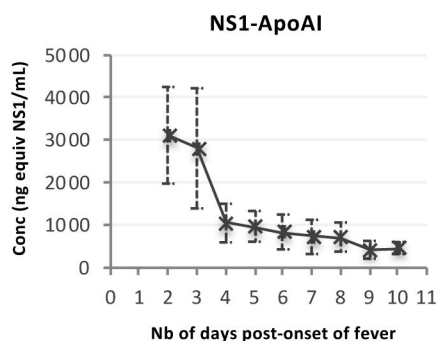
B



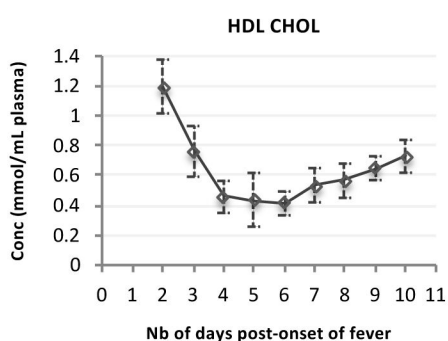
F



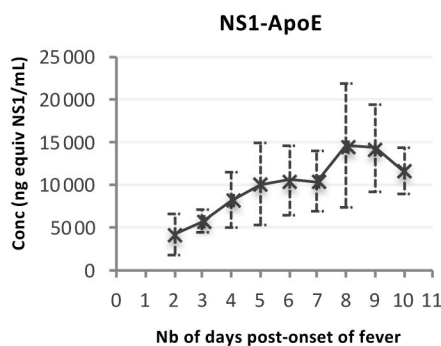
C



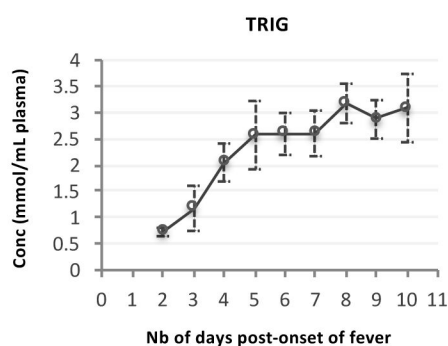
G



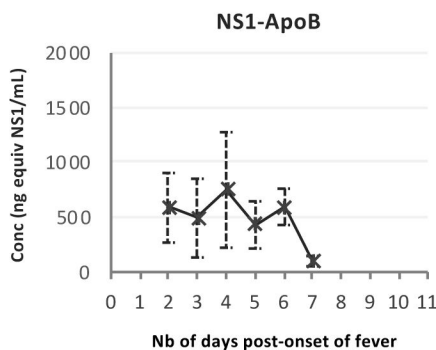
D



H



E



I

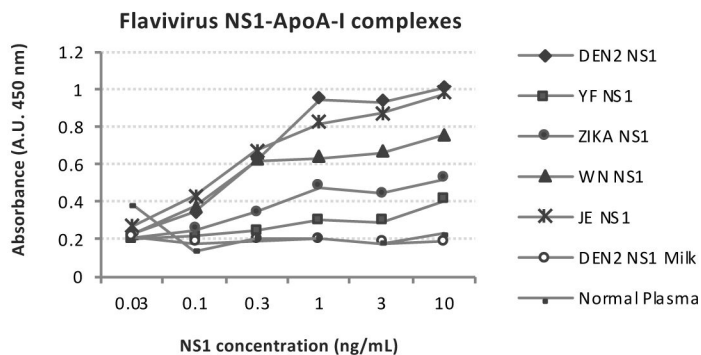
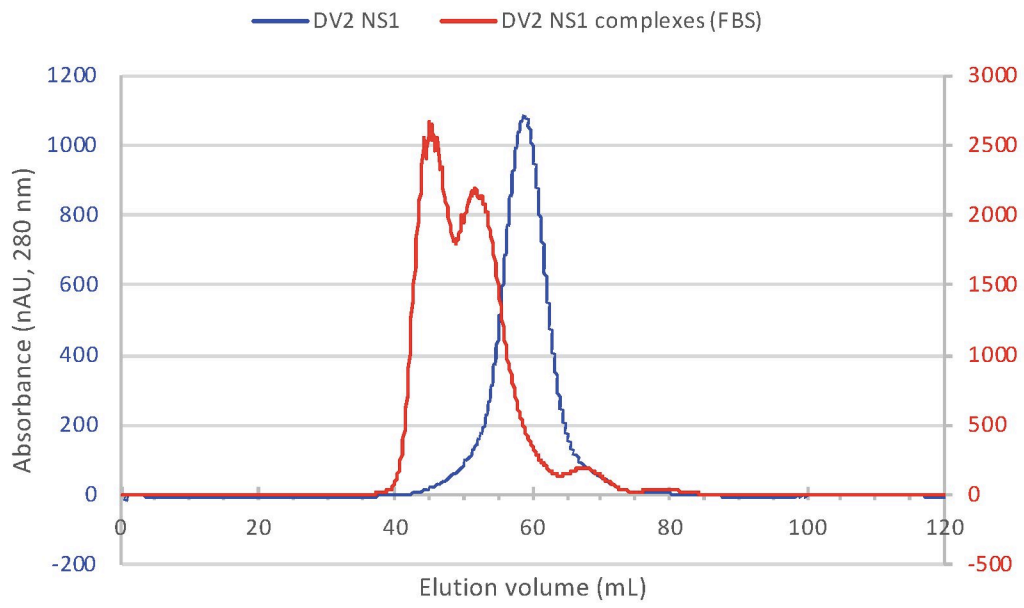
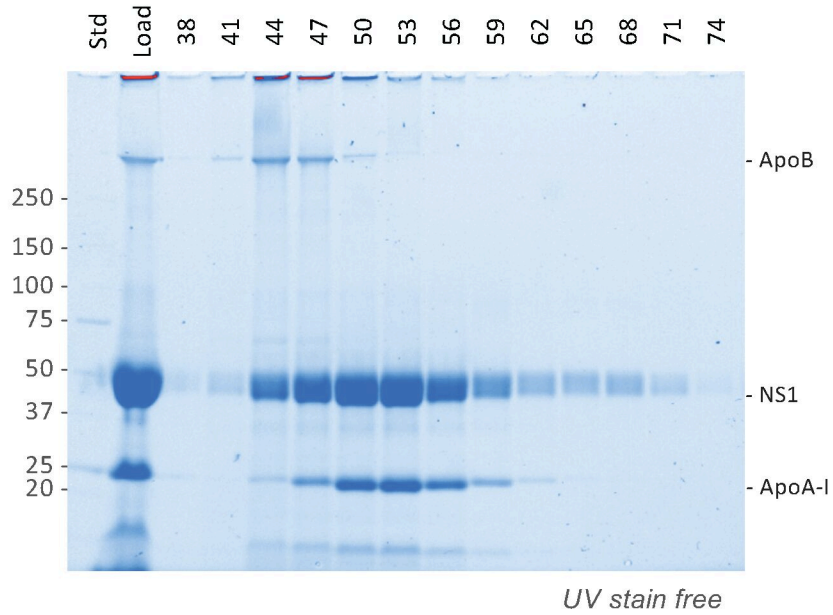


Fig. EV1

A



B



C

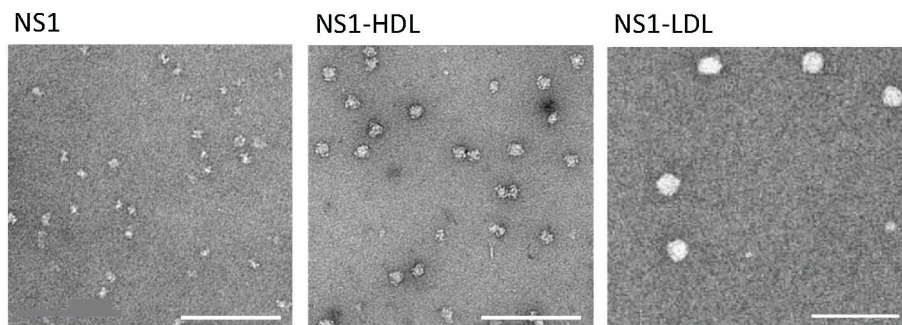
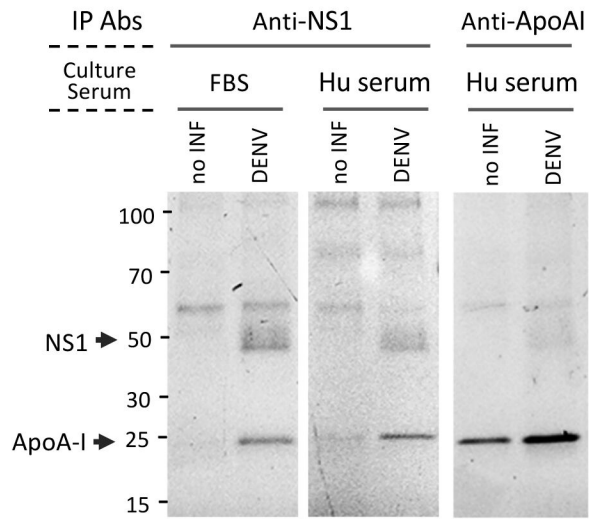




Fig. EV2

A



B

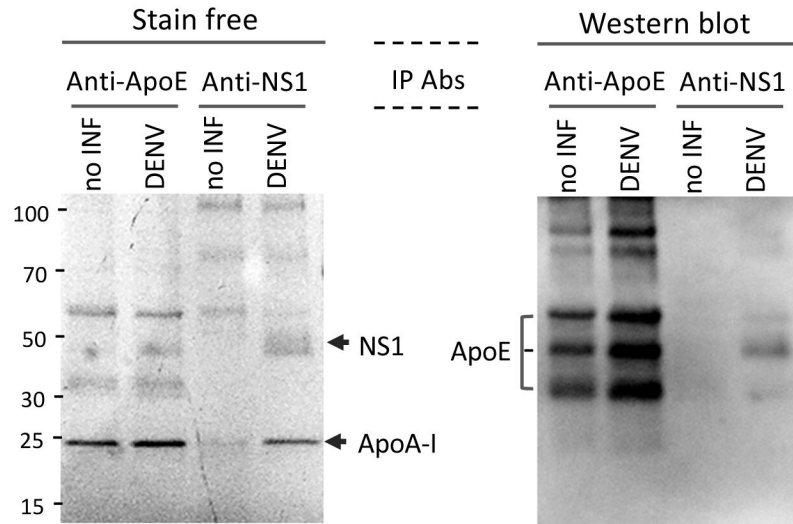


Fig. EV3

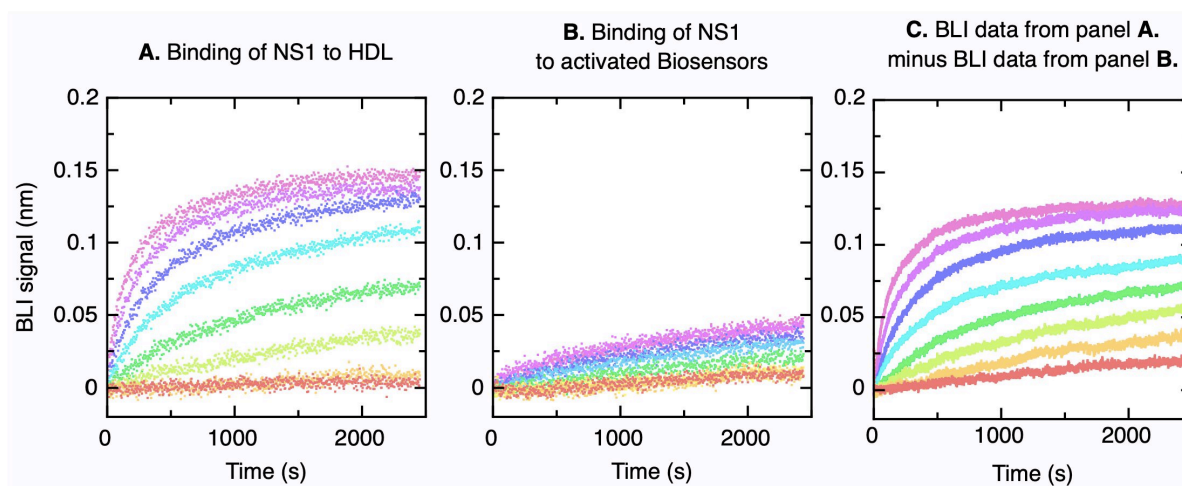


Fig. EV4

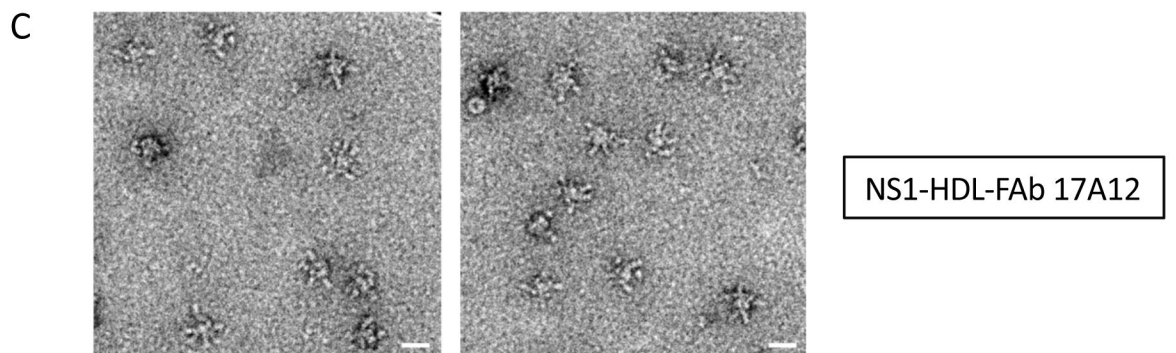
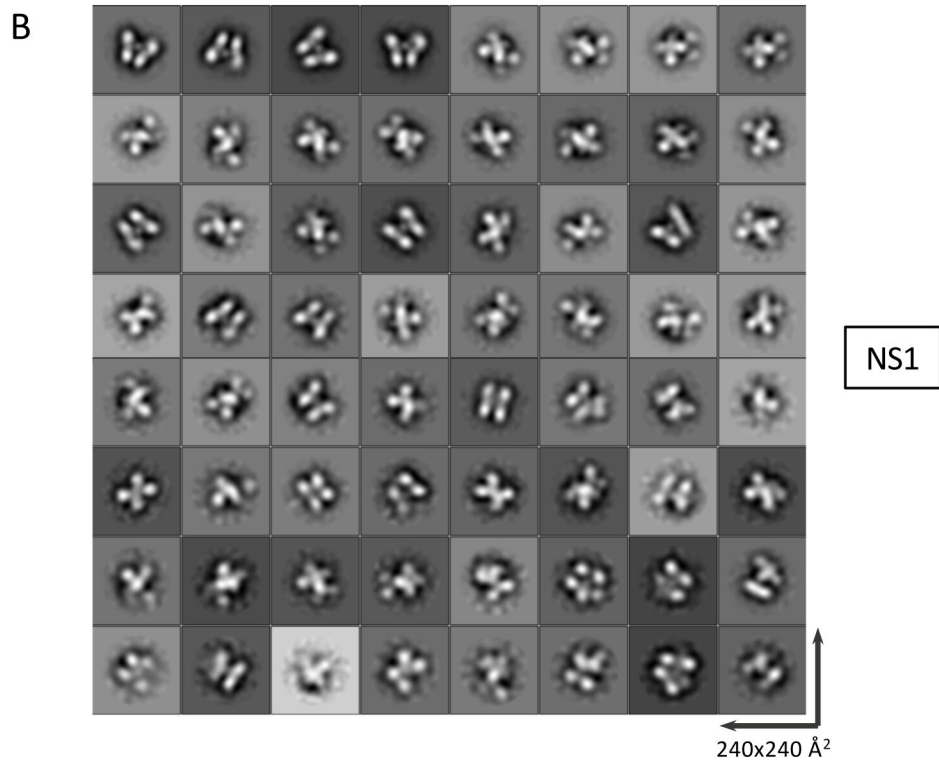
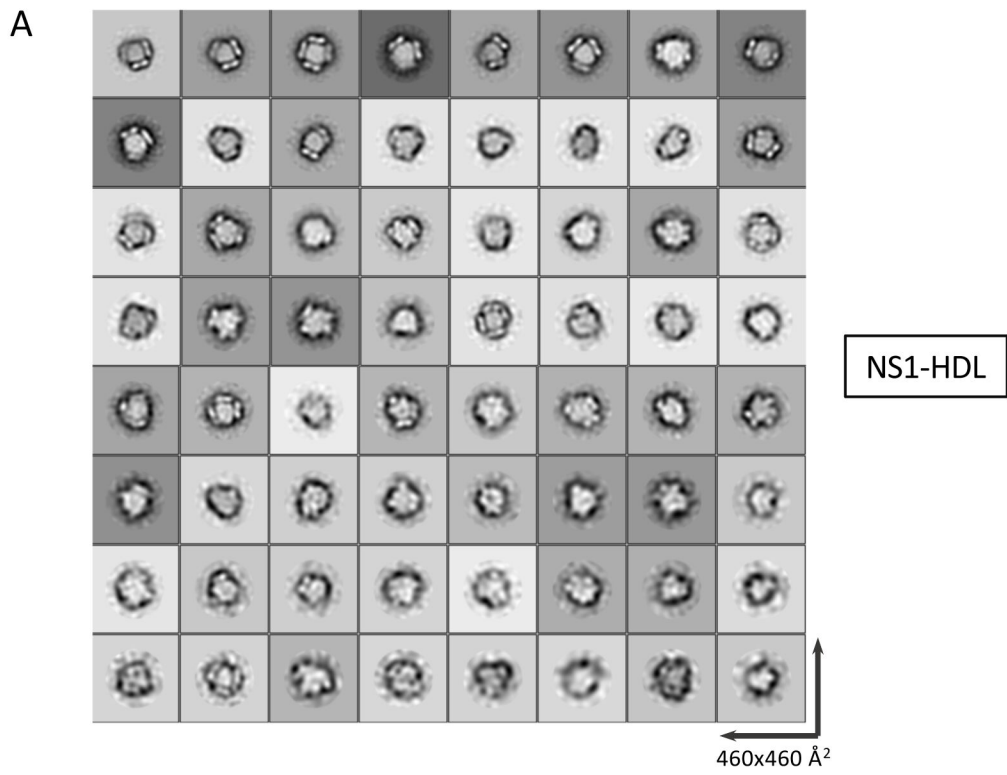


Fig. EV5

