The low-density lipoprotein receptor-related protein-1 is essential for Dengue virus infection

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Abstract

Dengue virus (DENV) causes the most prevalent and rapidly spreading arboviral disease of humans. It enters human cells by receptor-mediated endocytosis. Numerous cell surface proteins have been proposed as DENV entry factors. Among these, the phosphatidylserine receptor TIM-1 is the only one known to mediate virus internalization. However, several cellular models lacking TIM-1 are permissive to DENV infection, suggesting that other receptors exist. Here we show that the Low-density lipoprotein receptor-related protein-1 (LRP1) binds DENV virions by interacting with the DIII of the viral envelope glycoprotein. DENV infection is effectively inhibited by the purified receptor at $5x10^{-8}$ mol/L and the interaction of the envelope protein with LRP1 is also blocked by a natural ligand of LRP1. Depletion of LRP1 causes 100-fold lower production of infectious virus than controls. Our results indicate that LRP1 is another DENV receptor thus, becoming an attractive target to evaluate for the development of effective antiviral drugs against DENV.

Author summary

Dengue virus (DENV) is a complex of four related viruses, recognized as serotypes, designated as DENV1-4. Any of the four DENV serotypes can cause a self-limited disease of mild flu-like symptoms known as dengue or its life threatening form, severe dengue, with hemorrhagic manifestations, organ impairment and shock. This disease is

widely spread in tropical and sub-tropical areas worldwide, where the incidence of severe dengue has been increasing steadily. So far, efforts that target components of the viral replication machinery in order to develop a specific antiviral drug for dengue disease patients have failed. Thus, identifying the cell surface receptors used by DENV to enter host cells would provide a new molecular target to develop inhibitory drugs. Here, we evaluate the Low density lipoprotein receptor-related protein-1 (LRP1) as a putative DENV receptor. We present evidence demonstrating that LRP1 binds DENV through the viral envelope protein. We show that the production of infective virus is impaired on cells lacking LRP1, and that purified LRP1 is a potent blocker of DENV infection. These results are consistent with LRP1 playing an important role on DENV entry, making this receptor a molecule of interest on the investigation for medical treatments of dengue/severe dengue disease.

49 ## Introduction

50 The Dengue complex consists of four viral serotypes (DENV1-4) of a mosquito-borne
51 virus of the family *Flaviviridae*; genus Flavivirus. DENV-caused disease encompasses a
52 wide spectrum of clinical manifestations ranging from a mild self-limited disease known
53 as Dengue Fever to the life-threatening manifestations of Dengue Hemorrhagic Fever
54 [1]. The global incidence of DENV infections has grown dramatically in recent decades,
55 making this an arbovirus of worldwide public health concern [2].

56 DENV particles consist of a nucleoprotein core formed by RNA and the capsid protein.
57 This core is surrounded by a host derived lipid bilayer with two glycoproteins inserted:
58 the membrane (M) protein or its precursor (prM), found in immature virions and the
59 envelope (E) protein [3]. This latter is organized in three structural domains DI, DII and
60 DIII [4]. Of these, the DIII has been identified as directly involved on virus:receptor
61 interactions [5–8]. However, no specific receptor has been identified that binds the virus
62 through this region of the E protein.

63 DENV enters cell by receptor-mediated endocytosis [9]. Early steps of DENV infection
64 comprise interactions with soluble and membrane-bound attachment factors, signaling
65 receptors and internalization receptors [10].There are two main scenarios for these
66 events, the primary infection with any of the four virus serotypes and a secondary
67 heterologous infection [10]. In the latter, antibodies generated during the first infection
68 cross-reacting but not cross-neutralizing the infecting serotype act as soluble

69 attachment factors that bridge the virus to Fc receptors in the cell surface. Interaction
70 with Fc receptors also appears to facilitate virus internalization [11,12] and to generate
71 intracellular signaling that suppress intracellular antiviral responses [13,14]. All these
72 factors together lead to a significant increase in the number of infected cells and virus
73 production. This phenomenon, known as antibody dependent enhancement, results in
74 an increased susceptibility of infected individuals to the development of severe forms of
75 DENV-caused disease.

76 DENV also bind proteins such as Apolipoprotein A1 (ApoA1) [15] and Growth arrest-
77 specific protein 6 [16] that bridge the virus with cell surface receptors, the scavenger
78 receptor class B type I (SR-BI) and Tyrosine-protein kinase receptors Tyro 3, AXL and
79 MER (TAM), respectively. SR-BI and TAM receptors have been shown to increase virus
80 production but their role on early steps of infection has not been completely elucidated.
81 Other well characterized attachment factors that DENV binds on the cell surface are
82 heparan sulfate glycosaminoglycans [17], the C-type lectins DC-SIGN [18,19] and L-
83 SIGN [20] and the mannose receptor [21]. DC-SIGN and the mannose receptor have
84 also been shown to act cooperatively in DENV binding to the signaling receptor C-type
85 lectin domain family 5 member A (CLEC5A) [22]. This interaction enhances human
86 macrophage CLEC5A/DAP12 signaling as part of the innate immune response. Thus,
87 complexes formed by DENV attachment factors with signaling receptors may function in
88 favor or against the infection.

89 Internalization receptors mediate virus entry via receptor-mediated endocytosis which
90 ultimately leads to the delivery of the viral genome into the cytoplasm [23]. So far, only

91 the T-cell immunoglobulin and mucin domain 1 (TIM-1) receptor, which binds
92 phosphatidylserine moieties on the surface of the viral membrane, has been
93 convincingly shown to mediate DENV endocytosis [24]. However, some cellular models
94 that do not express TIM-1 are still sensitive, at low levels, to DENV infection. Therefore,
95 we have not reached yet a complete understanding of the process whereby DENV
96 gains entry into mammalian and mosquito cells, and additional molecules, binding either
97 the phospholipids of the viral surface or the E protein, may participate in receptor-
98 mediated endocytosis of this pathogen.

99 Regulation of lipid metabolism influences significantly the efficiency of DENV infection
100 and replication *in vitro* e *in vivo* [25–28]. Sequence stretches on DENV E and capsid
101 proteins resemble receptor binding motifs of ApoE [29]. ApoE is present in many
102 lipoproteins and interacts with several receptors of the low-density lipoprotein receptor
103 (LDLR) family [30]. Bovine lactoferrin, partially inhibits DENV and Japanese encephalitis
104 infection presumably competing for binding to the LDLR [31,32]. Collectively, these
105 results have suggested that LDLR may be involved on DENV entry to target cells. Yet,
106 conclusive evidences of the participation of LDLR on DENV attachment and/or entry
107 have not been obtained.

108 The LDLR family comprises at least 13 separate cell receptors that mediate lipoprotein
109 internalization, although some also play a role in the regulation of cellular physiology
110 and intracellular signaling [33]. In the context of the early events of the DENV infectious
111 cycle we decided to focus our interest on a different member of the family, the LRP1
112 receptor. This protein plays a role not only in lipoprotein endocytosis but also in the

113 internalization of protease:serpin complexes and, particularly of activated alpha-2-
114 macroglobulin (α2M*):protease complexes [34]. We have found that proteins
115 participating in these processes are significantly enriched within the plasma DENV
116 interactome [35,36]. Also we have shown that α2M*, a ligand of LRP1, directly binds
117 DENV virions and favors DENV infection [37].

118 Materials and Methods

119 Cells and viruses

120 Human hepatoma derived cells (Huh7) and human embryonic kidney cells (HEK-293T)
121 cells were grown under standard conditions at 37°C, 5% CO_2 in Dulbecco's modified
122 Eagle's medium (DMEM) + GlutaMAX supplemented with 10% of fetal bovine serum
123 (FBS), adding heat -inactivated FBS to a final concentration of 10% (v/v).

124

125 Proteins

126 Recombinant proteins DVE1 (code MBS144575), DVE3 (code MBS 144886) and DVE4
127 (code MBS 145224) were purchased from MyBioSource, Inc, USA. The procedures for
128 the expression, purification and general characterization of the proteins corresponding
129 to the domain III of the E protein of the four DENV serotypes namely DIIIE1-4, were
130 conducted as previously described. [37]. Receptor-activated α2M was purified from
131 human plasma also following the procedure described elsewhere [37].

132 The human protein associated to the LRP1 receptor, known as RAP was obtained by
133 expressing in *E. coli* a gene fragment coding for this molecule. The gene for RAP
134 protein was amplified from the cDNA by PCR using the oligonucleotides
135 **CATATG**TACTCGCGGGAGAAGAACCAG and **CTCGAG**TCAGAGTTCGTTGTGC,
136 bearing on their 5' end the recognition sequence for the restriction enzymes Nde I and
137 Xho I (in boldface in the sequence). The amplified fragment was used to construct pET-
138 RAP, a plasmid that codes for the intracellular synthesis of RAP under control of the T7
139 promoter. For protein expression, pET-RAP plasmid was transformed into the *E. coli*
140 strain BL21. Recombinant RAP protein was obtained with a tag of 6 consecutive
141 histidines at the N-terminus. RAP protein was purified from the supernatant by
142 immobilized metal affinity chromatography using Ni-NTA agarose and eluted with a
143 linear gradient of 10 to 300 mM imidazole in phosphate buffered saline (PBS, 10 mM
144 Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCL, pH 7.4).

145 Recombinant proteins sLRP1_CII, sLRP1_CIII and sLRP_CIV were purchased from
146 R&D Systems, USA. The Flavivirus-reactive mAb 4G2 was provided by the Unit for
147 Production of Monoclonal Antibodies at the Center for Genetic Engineering and
148 Biotechnology in Sancti Spiritus, Cuba. Mab MCA1965, was purchased from AbD
149 serotec, USA and Mab 5A6 was donated by Dr. D. K. Strickland.

150 **Affinity purification of LRP1**

151 Frozen human plasma was obtained from local blood banks. Pooled human plasma was
152 dialyzed against 50 mM Hepes pH 6, 60 mM NaCl, 1 mM EDTA, centrifuged at 10,000 x
153 g for 30 min at 4°C and filtered through 0.45 µm membrane. The resulting sample was

154 fractionated using anion exchange chromatography as previously described [38].
155 Briefly, conditioned plasma was loaded onto a chromatography column packed with DE-
156 52 gel previously equilibrated with the same buffer used for the dialysis. The sample
157 eluted by step-washing of the column with 50 mM Hepes pH 6, 1 mM EDTA and
158 increasing concentration of NaCL (0.3M, 6M and 1M) was then conditioned for loading
159 in the affinity chromatography using receptor-activated α2M as immobilized ligand.
160 Pooled elution fractions were dialyzed against 50 mM HEPES pH 7.0, 120 mM NaCl, 5
161 mM $CaCl_2$, 1 mM $MgCl_2$ and supplemented with a protease inhibitor cocktail (1 μg/ml
162 leupeptin, 1 μg/ml pepstatin A, 1 μg/mL soybean trypsin inhibitor and 1 mM PMSF).
163 Then, sample was loaded into a column packed with the affinity matrix at a flow of 10
164 cm/h. The column was washed with 100 column volumes of the loading buffer. Bound
165 protein was eluted with 50 mM HEPES pH 6.0, 1.2 M NaCl, 25 mM EDTA. Eluted
166 protein sample was dialyzed against the loading buffer, filtered through 0.2 µm and
167 stored at -20°C until used.

168 **Surface plasmon resonance analysis (SPR)**

169 SPR analyses were performed on a Biacore X unit (General Electric, USA). Proteins
170 were covalently immobilized on CM5 chips using amine coupling chemistry as
171 previously described [37]. DIIIE1 (30 µg/ml) and RAP (20 µg/ml) proteins were
172 dissolved in 10 mM sodium acetate buffer pH 5 and loaded into the system.
173 Immobilization of sLRP1_CIV was carried out at 10 µg/ml in 10 mM sodium acetate
174 buffer pH 4.0. All SPR experiments were performed at a flow rate of 25 µl/min using
175 Hepes 10 mM, pH 7.4, 0.15 M NaCl, 3 mM $CaCl_2$, 0.005% surfactant P20 (HBS-Ca) as

176 running buffer. The interacting surface was regenerated with a pulse of 5 µl of 10 mM
177 NaOH. Results were analyzed using the BIAevaluation ver. 4.1 software application
178 package (General Electric, USA).

179 **Pull down assays**

180 Purified DIIIE1-4 and RAP were immobilized at a ligand density of 4 mg/mL in CNBr-
181 activated Sepharose (Amersham, UK), as recommended by the provider. A background
182 control matrix was prepared following the identical procedure but without any
183 immobilized protein.

184 To obtain the microsomal fraction from Huh7 cells, subconfluent monolayers were
185 scraped from the flasks, washed three times with PBS, resupended in 1 mL of hypotonic
186 buffer (20 mM Tris pH 7.4, 10 mM KCl, 5 mM EDTA, 1 mM PMSF, 1 µg/mL peptstatin,
187 1 mM leupeptin) and incubated 20 min on ice. All subsequent steps were performed at
188 4°C. Sucrose was added to a final concentration of 250 mM, the cell suspension was
189 transferred to a Dounce homogenizer and disrupted by 3 strokes followed by a brief
190 sonication. Cell homogenates were first centrifuged at 1 000 x g to remove nuclei and
191 supernatants were then centrifuged at 10 000 x g for 20 min to pellet mitochondrial
192 fraction. The microsomal fraction was obtained by centrifuging post-mitochondrial
193 supernatant at 100 000 x g for 1 h. The resulting 100 000 g pellet was resuspended at
194 10 mg/ml in HBS-Ca and stored at -80 ºC until use.

195 The microsomal fraction was split in 6 identical aliquots, each of which was incubated
196 with 1 mL of the affinity matrices and background control gel for 18 h at 4°C in a rotary

197 mixer. The affinity gels were washed with 100 matrix volumes of HBS-Ca, 0.005% n-
198 Octyl-β-D-Glucopyranoside. Bound proteins were eluted by incubating the gels twice for
199 15 min at 25°C with 2 mL of 0.1 M acetic acid followed by 1 mL of 3 M urea, 1 mM
200 EDTA. The three eluates from each gel were then pooled, concentrated and adjusted to
201 pH 8.0 by adding 0.2 mL of Tris-HCl 2 M, pH 8.0. Protein digestion, Selected Reaction
202 Monitoring analysis and data processing were performed as described [35].

203 **Virus assays**

204 Both viruses DENV2 (strain S16803, NIBSC code S16803) and Vesicular Stomatitis
205 Virus (VSV strain Indiana) were propagated and titrated by plaque formation assays
206 performed in Vero cells. Plaque formation assays were performed in 24 well plates with
207 cell monolayers at 90% of confluence. Serial dilutions of virus inoculum were diluted in
208 DMEM 2 % FBS, added to cells and incubated for 2 h in the CO_2 incubator. Next, viral
209 dilutions were removed, cell monolayers were washed twice with DMEM 2% FBS and
210 incubated for 5 days at 37°C in high density medium (MEM supplemented with non-
211 essential amino acids, 1% FBS, 1% carboxymethylcellulose) in order to propitiate the
212 appearance of lytic plaques. The plaques were visualized by staining with 0.1% Naphtol
213 Blue Black in 0.15 M sodium acetate. Two replicates were used per experimental point
214 in each assay.

215 For the purification of DENV2, monolayers of Vero cells were infected at a moi 0.01,
216 wash with fresh DMEM without FBS and maintained in serum free medium for 96 hrs.
217 Cell supernatants were clarified by centrifugation at 5000 rpm for 30 min. Virus were
218 sedimented by ultracentrifugation at sucrose 30% in 5 mM HEPES, 150 mM NaCl, 0.1

219 mM EDTA at 32 000 rpm for 9 hours. Virus pellets were resuspended in the same buffer
220 without sucrose. The purified virus preparation was analyzed by SDS-PAGE followed by
221 Coomasie blue staining. Visible protein bands corresponded to E and pre-M proteins as
222 confirmed by western blotting using specific Mabs for each protein.

223 Inhibition of infection with chromatography fractions were performed using a plaque
224 formation assay in Huh7 cells. Cells were seeded at 10^4 per well in 24-well plates and
225 kept for 18 h at 37°C in a CO_2 incubator. DENV2 inoculum was diluted in DMEM without
226 FBS and adjusted to obtain 50 PFU/well. Chromatography fractions were diluted in
227 DMEM without FBS, added to the virus inoculum to obtain a final protein concentration
228 of 10 µg/mL and incubated for 30 min at 37°C. For virus control, only PBS was added to
229 the virus inoculum previous to the incubation. Next, virus-sample mixtures were added
230 to cell monolayers and infection proceeded for 2 h at 37°C. Afterwards, cell monolayers
231 were washed and high density medium was added. Viral plaques were visualized 5
232 days post-infection.

233 For the evaluation of virus yield after transfection with shRNAs, Vero cells were seeded
234 in 96 well plates and cultured until monolayers reached 90% of confluence. Viruses
235 were diluted in DMEM, 2% FBS for an m.o.i of 0.1 in case of DENV2 and 0.35 for VSV.
236 Monolayers were washed once with DMEM 2% FBS and subsequently 100 µl of virus
237 inoculum were added. After 1 h incubation viral inoculum was removed and replaced by
238 100 µl of fresh DMEM 2% FBS. To evaluate viral production, 24 h supernatant was
239 collected and assayed for infectious virus by plaque assay on Vero cells.

240 **Gene silencing**

241 Target sequence within the hLRP1 mRNA (5'-GATCCGTGTGAACCGCTTTAA-3')
242 (NM_002332.2, RNAi Consortium at http://www.broadinstitute.org/rnai/public) was
243 confirmed to match within a CDS on the LRP1 gene sequence and to be unique for this
244 gene. Template sequences encoding a short hairpin RNA were generated and cloned
245 into de pKLO.1-TRC shRNA lentivector:

246 Forward oligo:

247 5'-
248 CCGGGATCCGTGTGAACCGCTTTAACTGCAGTTAAAGCGGTTCACACGGATCTTTT
249 TG-3'

250 Reverse oligo

251 5'-
252 AATTCAAAAAGATCCGTGTGAACCGCTTTAACTGCAGTTAAAGCGGTTCACACGGA
253 TC-3'

254 Oligonucleotides (Integrated DNA Technologies, USA) were annealed, phosphorylated
255 and ligated into AgeI and EcoRI sites of the pKLO.1 vector. Plasmid containing clones
256 were selected by resistance to ampicillin and checked by restriction analysis and
257 sequencing.

258 Monolayers of 293T cells were co-transfected with packaging construct (psPAX2)
259 coding for gag, pol, tat and rev of HIV; envelope plasmid (VSV-G) and pLKO.1 based
260 shRNA-LRP1. As negative controls were used two pLKO.1 constructs designed to

261 activate the silencing machinery but without targeting any gene in the cells: shRNA-
262 scramble. Transfection was performed in 10 cm plates by the Ca-phosphate method
263 using 10 µg of DNA mixed in a ratio pLKO.1_shRNA:psPAX:VSV-G 4:3:1. After 24
264 hours, media containing transfection mixtures was replace by fresh DMEM, 10% FBS.
265 Lentivector containing supernatants were harvested at 48 and 72 hours post-
266 transfection. Collected supernatants were clarified by centrifugation followed by filtration
267 through 0.45 µm, aliquoted and store at -80°C until used.

268 Lentivirus containing supernatants were used at indicated dilutions to infect Huh7 cells.
269 Infection was performed in DMEM, 10% FBS, 5 µg/ml polybrene. After 18 h lentivirus
270 inoculum was removed and replaced by selection medium: DMEM, 10% FBS, 1 µg/ml
271 Puromycin (InvivoGen, USA). Efficiency of transduction was estimated by detection of
272 GFP expression using FACS.

273 Huh7 cells transduced with either the LRP1-specific shRNA or a scrambled shRNA
274 control were infected at day 7 post-transduction with DENV2 and infectious particles
275 release was quantified by titration 24 h post-infection.

276 **Denaturing polyacrilamide gel electrophoresis (SDS-PAGE)**

277 One microgram of protein of chromatography fractions was diluted in non-reducing
278 sample buffer, loaded in a 6-15% gradient polyacrylamide denaturing gel and separated
279 under standard conditions [39]. Proteins were detected using silver staining [40]. For the
280 evaluation of LRP1 expression after gene silencing 10^5 cells were lysed in non-reducing

281 sample buffer, separated in an 8% polyacrylamide gel and subjected to western blotting
282 analysis.

283 **Western blotting**

284 Separated proteins were transferred to nitrocellulose membranes under standard
285 conditions [41]. After transfer membranes were incubated for 1 hr at 25°C with Hepes
286 20 mM pH 7.2, 0.05% tween 20, 1 mM $CaCl_2$. Membranes were then blocked for 1h
287 using 1% Bovine Serum Albumin (BSA) and incubated with a 10 µg/ml dilution of
288 primary antibody for 16 hrs at 4°C. Next, blots were washed three times with PBS-T and
289 antibody binding was detected using an anti-mouse antibody conjugated to HRP. For
290 visualization, ECL development solution was used in the evaluation of LRP1 expression
291 after gene silencing and 3,3'-diaminobenzidine (Sigma, USA) in the analysis of affinity
292 chromatography fractions.

293 **ELISA**

294 Ninety-six well microtiter plates were coated for 1 h at 37°C with 50 µL per well of the
295 corresponding protein at 10 µg/ml or $2x10^6$ pfu of purified DENV2 in carbonate buffer
296 pH 9.6. The plates were blocked with 2% (w/v) BSA in PBS, 0.05% Tween 20 (PBS-T)
297 for 1 h at 37°C. Recombinant sLRP-CI-IV were diluted in HBS-Ca, added to coated
298 wells and incubated 1 h at 37°C. After three washes with binding buffer, bound proteins
299 were detected using an anti-human Ig, Fc specific-HRP conjugate. The development
300 was carried out with 1 mg/ml o-phenylenediamine dihydrochloride, 0.1% hydrogen

301 peroxide in 0.05 M phosphate-citrate buffer, pH 5.0. Developing reaction was stopped
302 with 3 M H_2SO_4 and results were read at 492 nm in a microplate reader.

303 **Statistical Analysis**

304 GraphPad Prism V5.3 was used to perform unpaired Student's t-test to calculate p
305 values [42]. Differences with P-values of <0.05 were considered significant (*p<0.05,
306 **p<0.01, ***p <0.001).

307

308 ## Results

309 **LRP1 depletion inhibits DENV2 infection**

310 To investigate whether the presence of LRP1 on the cell surface could impact the
311 efficiency of infection, we examined the effect of sh-RNA mediated LRP1 knock-down
312 on viral yield. The assay was performed in Huh7 hepatoma cells, which are extensively
313 used in *Flaviviridae* research, physiologically relevant for DENV infection [43] and
314 express LRP1 [44]. Huh7 cells can be efficiently transduced by lentiviral vectors without
315 alterations to key hallmarks of the hepatic phenotype [45]. Western blotting analysis
316 revealed that LRP1 expression was efficiently inhibited by day 6 post-transduction (Fig
317 1A). Viral yield in the cells transduced with the LRP1-specific shRNA was 100-fold lower
318 than in the cells transduced with the scrambled control (Fig 1B). These results show
319 that LRP1 plays a central role on DENV replication cycle. Meanwhile, VSV which

320 presumably can use LRP1 as an alternative to LDLR [46], significantly less efficient than
321 this latter, exhibits only a slight decrease in virus titer.

322 **Fig 1. LRP1 is essential for DENV2 infection.**

323 (A) Western blotting analysis of the silencing of LRP1 expression on cells transfected
324 with scramble shRNA (CS) or with LRP1-shRNA. LRP1 was detected using the Mab
325 5A6 that recognizes the 85-kDa β chain of the receptor [47]. Detection of βactin was
326 performed on the same membrane. (B) Effect of the transduction of LRP1-shRNA on
327 DENV2 infection. Transduced cells were infected at day 7 with DENV2 (moi of 0.1) or
328 Vesicular Stomatitis Virus (VSV, moi 0.35). Virus yield was measured on 24 hr post-
329 infection supernatants.

330

331 **Ligand-purified LRP1 inhibits DENV2 infection in hepatocytes**

332 One testable prediction of the hypothesis that LRP1 participates on early steps of
333 virus:cell interaction would be to inhibit DENV infection by competition with a soluble
334 form of LRP1. Therefore, we purified the LRP1 ectodomain (sLRP1) from human
335 plasma by using receptor-activated α2M as an affinity ligand (Figs 2A-C), taking
336 advantage of the fact that under physiological conditions sLRP1 is shed from cell
337 surfaces into body fluids in a soluble, active ligand-binding form [48]. SPR experiments
338 with the purified sLRP1 on chips containing immobilized RAP, which is the intracellular
339 receptor chaperone of LRP1 [49], confirmed that it retained its normal ligand-binding
340 activity (Fig 2D).

341 **Fig 2. Purified sLRP1 inhibits DENV2 infection.**

342 (A) Chromatographic profile of the affinity chromatography using receptor-activated α2M
343 as immobilized ligand. PT: pass-through. Numbers in parenthesis identify the lane in
344 SDS-PAGE (B) and western blotting analysis. In (C) membrane was probed with an
345 anti-LRP1 Mab (MCA1965) that recognizes the alpha chain of the receptor. (C).
346 Microsomal fraction (FM) of Huh7 cells was used as control of migration of α chain of
347 LRP1. (D) Binding analysis of purified sLRP1 to recombinant RAP protein using SPR.
348 RAP protein was immobilized on the surface of the chip and purified sLRP1 was
349 injected diluted in HBS-Ca at 1 µg/mL. (E) Inhibition of DENV2 infection in Huh7 cells
350 after incubation with chromatography fractions. Results from one representative
351 experiment out of three independent experiments. Error bars represent the range of
352 three sample replicates.

353

354 An inhibition-of-infection assay was then set up in Huh7 cells where a DENV2
355 preparation was pre-incubated either with purified sLRP1 or with different fractions
356 collected during the purification process (the initial sample, the pass-through and the
357 washing eluate). As previously reported, on these experimental conditions the human
358 plasma fraction used as starting sample shows partial inhibitory activity against DENV2
359 infection of Huh7 hepatocytes [50]. Neither the pass-through nor the washing eluate
360 exhibited inhibitory activity, while the eluate containing sLRP1 at $5x10^{-8}$ mol/L, exhibited
361 an inhibitory activity close to 90% indicating a significant enrichment of inhibitory activity

362 (Fig 2E). These results indicate a direct interaction of LRP1 with DENV2 and are
363 consistent with the involvement of LRP1 in the initial stages of the DENV life cycle. The
364 high potency of the observed inhibitory effect suggests that it involves the blockage of
365 critical functional sites on the viral surface (*i.e.* direct competition with cell-bound LRP1)
366 rather than the indirect occlusion of binding sites for other cell receptors.

367 **LRP1 interacts with DENV particles**

368 LRP1 is a heterodimeric type I integral membrane protein comprised of an
369 extracellular 515-kDa α chain and a non-covalently linked cytoplasmic and
370 transmembrane 85-kDa β chain (Fig 3A). The interaction of LRP1 with extracellular
371 ligands typically takes place through the complement-type repeats that are individually
372 known as ligand binding domains (LBD) and are presented in four clusters of 2, 8, 10
373 and 11 LBD on the α chain denominated CI, CII, CIII and CIV, respectively. Of these,
374 clusters CII-CIV are those that bind most of LRP1 ligands [49].

375 **Fig 3. Interaction of DENV2 with ligand binding clusters of LRP1.**

376 Schematic representation of (A) LRP1 and (B) the recombinant proteins sLRP1-CII,
377 sLRP1-CIII and sLRP1-CIV. (C) Evaluation of the interaction of recombinant proteins
378 sLRP1-CII, sLRP-CIII and sLRP1-CIV with purified DENV2. (D) Interaction of sLRP1-
379 CIV with recombinant preparations of the ectodomain of the E protein of DENV
380 serotypes 1, 3 and 4 (DENVE1, DENVE3 and DENVE4) by SPR. sLRP1-CIV was
381 immobilized in the surface of the chip and recombinant DENV E proteins were injected
382 diluted in HBS-Ca at 10 μg/mL. Resp Diff. Signal obtained by online subtraction of

383 background control channel. (E) Binding competition by ELISA. Plates were coated with
384 DENVE1 protein and the binding of sLRP1-CIV at 5 µg/ml was evaluated after the
385 incubation for 30 min at 37°C with EGF or the LRP1 ligand protein RAP, both proteins
386 used at 5 µg/ml. BC: background control. Results are representative of two independent
387 analyses. Error bars represent the range of three replicates.

388

389 In order to investigate whether DENV particles can bind LRP1, we analyzed the
390 interaction of a purified preparation of DENV2 adsorbed to ELISA plates with three
391 chimeric proteins denoted sLRP1_CII, sLRP1_CIII and sLRP1_CIV, containing clusters
392 II, III or IV from LRP1 fused to a human IgG1 Fc domain (Fig 3B). As shown in Fig 3C,
393 DENV2 bound efficiently the fusion protein containing cluster IV, but not those
394 containing clusters II or III. Considering that the construct of sLRP1-CIV protein
395 comprises only the 11 LBD of CIV (Fig 3B), these results directly imply that, similar to
396 the interaction with other extracellular ligands of LRP1, the complement-type repeats
397 (Fig 3A) are involved in the interaction with DENV2.

398 **The E protein of DENV binds LRP1**

399 The E protein covers most of the solvent-accessible surface of DENV virions
400 [51]. Therefore, we next investigated if protein E mediates the interaction between
401 DENV and LRP1 and whether this interaction is conserved for the other viral serotypes.
402 To this end, we examined by SPR the binding of recombinant proteins encompassing
403 the E ectodomain from DENV serotypes 1, 3 and 4 (DVE1, DVE 3 and DVE 4 in Fig 3D)

404 to sLRP1_CIV immobilized on an SPR chip. All three recombinant E proteins bound the
405 sLRP1_CIV-derivatized surface obtaining almost three times higher signal for DVE1
406 followed by DVE3 and DVE4 (Fig 3D). This result demonstrate that the DENV-LRP1
407 cluster IV interaction is mediated by protein E and that it is preserved in serotypes other
408 than DENV2, although with probable differences in reactivity among the four viral
409 serotypes. DVE1 was then selected to test whether RAP protein was able to block the
410 interaction using an ELISA format. Results showed that similar to many other LRP1
411 ligands, pre-incubation of sLRP1-CIV with RAP abolished the interaction with DVE1
412 while no effect was observed when another protein non-relevant for this system *i.e*. the
413 human Epidermal Growth Factor (EGF), was used as competitor (Fig 3E).

414 **The interaction of E with LRP1 is mediated by DIII of protein E**

415 A number of results indicate that DIII of E protein of Flaviviruses participate on
416 the interaction with cell surface molecules that play key roles in virus binding
417 [5,7,8,52,53]. We thus decided to investigate whether the protein E-LRP1 interaction is
418 mediated by this region of E protein. For this purpose, we used recombinant DIII
419 proteins of the four viral serotypes, denoted DIIIE1, DIIIE2, DIIIE3 and DIIIE4 (residues
420 289-400 of DENV1, 2, 4 and 287-388 of DENV3) (Fig 4A), to coat ELISA plates, which
421 were probed with the sLRP1-derived chimeras (Fig 3B). DIIIE from all four serotypes
422 bound sLRP1-CIV (Fig 4B). DIIIE2 also bound sLRP1_CII weakly, although this
423 reactivity was undetectable for the other three serotypes, and no binding to sLRP1_CIII
424 was detected for any of the recombinant DIII preparations. In this assay format, the
425 recombinant protein corresponding to DIII of DENV1 (DIIIE1) was the one showing the

426 lowest reactivity. This apparent contradiction with the results obtained by SPR with
427 DENVE1, 3 and 4 (Fig 3D) may reflect a higher susceptibility of the recombinant DIIIE1
428 protein to loose reactivity when adsorbed to the surface of the plate. In fact, DIIIE1
429 showed similar reactivity with purified sLRP1 as DIIIE2 when compared in SPR (Fig4C).
430 Specificity of the binding to DIIIE1 surface was evidenced using competition assay with
431 two LRP1 ligands, RAP and receptor activated α2M. As shown in Fig 4C binding to
432 DIIIE1 surface decreases significantly when RAP and receptor activated α2M were
433 incubated with sLRP1 before the analysis.

434 **Fig 4. Interaction of recombinant DENV E DIII proteins with LRP1.**

435 (A) Multiple sequence alignment of DIIIE1-4 proteins (residue numbers according to
436 DENV1, 2 and 4). The alignment was performed using the ClustalW application [54].
437 The arrows denote β-strands. Residues are colored according to the physico-chemical
438 properties and conservation according to ESPript program [55]. (B) Interaction of
439 DIIIE1-4 proteins with sLRP1-CII, sLRP1-CIII or sLRP1-CIV by ELISA. Results are
440 mean and SEM of three independent experiments. (C) Binding analysis of sLRP1 to
441 immobilized DIIIE1 by SPR. sLRP1 at 1 µg/ml was loaded over the DIIIE2 and DIIIE1
442 surfaces. For this later, sLRP1 injection was performed without or after pre-incubation
443 with RAP at (DIIIE1/RAP), or receptor-activated α2M (DIIIE1/α2M*). Resp. Diff.: non-
444 specific signal was online subtracted using a reference channel without any immobilized
445 protein. (D) Blocking of sLRP1-DENV2 binding. sLRP1-CIV at 5 µg/ml was incubated
446 with 10 µg/ml of DIIIE1-4 for 1 h before addition to DENV2 coated plates. Bound sLRP1-
447 CIV was detected using an anti-human Fc Ig-POD conjugate. (E) Binding to LRP1

448 present in the microsomal fraction of Huh7 cells. Pull down assay using recombinant
449 DIIIE1-4 proteins as baits and detection by Mass spectrometry using Selected Reaction
450 Monitoring. MF: microsomal fraction. BC: background control. Data shows transition
451 signals of the surrogate peptide $_{365}$IVFPHGITLDLVSR$_{378}$ (Swissprot Accession number
452 Q07954). Samples were analyzed in duplicate. Similar results were obtained for other
453 five proteotypic peptides (S1 Table). (F) Cartoon representation of the 3D structure of
454 DIII. In yellow are represented the β-strands and lysine residues are shown in wire-
455 frame representation. Residue numbers correspond to DENV1, 2 and 4.

456

457 The ability of recombinant DIII proteins to block the binding of sLRP1-CIV to
458 DENV2 (Fig 4D) was also evaluated. In the assay, DIIIE1-4 proteins were incubated in
459 solution with sLRP1-CIV before its addition to DENV2 particles adsorbed in ELISA plate
460 wells. The results show that DIII recombinant proteins of the different viral serotypes are
461 able to block sLRP1-CIV to DENV2 to different levels with more than 95% of inhibition
462 exhibited by DIIIE4 at the maximal protein concentration evaluated in the assay (100
463 μg/ml) followed by DIIIE2 > DIIIE1 > DIIIE3. This result shows that DIII is a dominant
464 site of interaction, although it does not rule out the existence of other regions within the
465 E protein that may contribute to the binding to sLRP1-CIV. This result also highlights the
466 fact that DIII surface patches involved on the interaction with LRP1 are sufficiently
467 similar that a recombinant DIII from one serotype can effectively compete for LRP1
468 binding to the virus particle of a different serotype.

469 The binding of recombinant DIII to full-length LRP1 was evaluated in pull-down
470 assays (Fig 4E). To this end, DIIIE1-4 were used as baits and incubated with a
471 microsomal fraction obtained from Huh7 cells, detecting bound LRP1 by selected
472 reaction monitoring in mass spectrometry. In this assay setup, both DIIIE2 and DIIIE4
473 were able to pull down the native LRP1 receptor as presented in the cell surface (Fig 4E
474 and Table A in S1).

475 Altogether, these results demonstrate that the four serotypes of DENV bind the
476 LRP1 receptor and that DIII mediated interaction is probably primary in binding to
477 cluster IV of ligand binding domains of this receptor. Even considering a possible
478 influence of different assay formats, DIII mediated interaction is stronger with serotype 4
479 followed by serotype 2 and weaker with serotypes 3 and 1.

480 Similar to other members of the family of the LDLR, LRP1 binding to its ligands
481 has been proposed to occur through the acidic necklace model: amino groups of lysine
482 residues of the ligands are encircled in a tripartite salt bridge via three remaining oxygen
483 atoms from the acidic residues forming the octahedral calcium cage of LBDs [56]. It
484 seems that there are modest requirements for the coordination of lysine residues [57],
485 but specificity may be influenced by other residues located in its vicinity showing
486 positive/negative charge [58,59] or hydrophobic character [60]. High affinity binding
487 seems to involve the interaction with two or more LBDs requiring at least two lysine
488 residues located at an appropriated distance - 18-20 Å according to structural data –
489 which allows the simultaneous binding of two consecutive LBDs [56]. Likewise, the
490 relative contribution of a single lysine residue of a ligand protein to the interaction may

491 be difficult to rationalize in terms of stereo-chemical properties and the overall binding
492 affinity may be the result of the additive effect of various weak binding sites [61].

493 The DIII contains 10-13 lysine residues depending on the serotype, but the
494 number of basic residues is counted by the sum of aspartic and glutamic acids resulting
495 in the fact that at neutral pH the overall charge is predicted to be minimal for DENV1-
496 DENV3 and only approximately +2 for DENV4 (Table B in S1). This observation
497 highlights the relevance of specific interactions involving lysine residues of DIII which
498 interact with the receptor according to the acidic necklace model. The overall positive
499 electrostatic potential of DIII from DENV4 may also contribute favorably to the
500 interaction and explains in part why this serotype displays the higher binding affinity.
501 Five lysine residues (K291, K295, K310, K334 and K394) are strictly conserved – in
502 sequence – among the serotypes but others are conserved topographically *i.e.* the
503 mutation of one lysine is accompanied by a compensating mutation to lysine in an
504 adjacent position in the 3D structure, e.g. the mutation K388 (DENV1) to T (DENV4) is
505 compensated by the mutation G344 (DENV1) to K (DENV4). Furthermore, there are
506 several interatomic distances between the lysine residues of DIII which are consistent
507 with a putative interaction with two consecutive LBDs. Otherwise, the sequence identity
508 among the DIII from the four serotypes varies between 52 and 71%, indicating that
509 mutations close in sequence and/or 3D space to lysine residues would also affect the
510 interaction and explain observed differences in binding affinity (Fig 2A and Table C in
511 S1).

512 ## Discussion

513 Viral tropism is strongly determined, to a great extent, by the specific host cell
514 receptor(s) leveraged by a virus to infect a specific cell or tissue type [62]. Thus, the fact
515 that DENV infects a dissimilar array of cell types including endothelial cells, fibroblasts,
516 myeloid cells and lymphocytes [63] might be explained by the use of a single,
517 ubiquitously expressed receptor or of several, varying cell receptors differentially
518 expressed in distinct cell lineages.

519 A substantial number of different cell surface molecules have been shown to be
520 involved in early stages of the DENV:cell interaction; most of these cases concern the
521 first step of the cell entry process, that is, adhesion [64]. The higher efficiency of
522 infection afforded by the ectopic expression of DENV adhesion molecules, such as DC-
523 SIGN, illustrates the importance of this initial interaction for the viral life cycle [18].

524 However, the following step, which in DENV case is cell entry by receptor-mediated
525 endocytosis, is as important as the first. In the case of human cells, receptor-mediated
526 endocytosis places the virion at the right cellular location and under the best conditions
527 for membrane fusion [23]. Although TIM-1 is the only molecule that has convincingly
528 been shown to internalize DENV by receptor-mediated endocytosis [24], both the
529 experimental results of the characterization of TIM-1 activity during endocytosis [16,24]
530 and the expression profile of this protein suggest that the virus must leverage other as
531 yet unknown molecules capable of receptor-mediated endocytosis.

532 For a cell receptor to be used as a viral port of entry, it must be able to bind the virus
533 directly or through a bridging ligand. Our results show that LRP1 binds DENV virions
534 (Fig 3C) and that this interaction competes, with potency at the nanomolar range, with
535 DENV virion interactions involving other cell surface molecules (Fig 2E). Previous
536 research has already shown that DENV virions can also bind LRP1 ligands [36,65,66],
537 and in the case of α2M, it has been proven that this interaction augments infection [65].
538 Whether DENV infection may proceed through the formation of a ternary
539 DENV:α2M:LRP1 complex is the subject of ongoing research, but we would like to point
540 out that the pathogenicity of WNV, a flavivirus closely related to DENV, is severely
541 reduced in knockout mice defective for the expression of α2M homologs [67]. Lastly, it is
542 worth noting that the assembly of ternary complexes is not exclusive to multivalent
543 LRP1 ligands such as DENV and α2M, but has been observed for other LRP1 ligands
544 as well [68].

545 LRP1 is a highly efficient constitutive endocytic receptor mediating the internalization of
546 over 40 different ligands, including viruses [49]. A direct interaction with this receptor
547 leads, with high probability, to the internalization of the ligand. Consequently, our results
548 demonstrating that the expression of LRP1 in human hepatocytes is a requisite for
549 efficient DENV infection in these cells (Fig 1) indicates that LRP1-mediated cell entry
550 must result in a productive infection event.

551 Severe dengue patients have been shown to exhibit reduced LRP1 expression levels
552 [69]. Based on this result, a proposal was put forth to include LRP1, together with
553 another 17 genes, into a molecular signature for the prognosis of dengue disease [69].

554 In light of our findings regarding the direct interaction of DENV virions with LRP1, this
555 proposal reinforces the notion that LRP1 is directly linked to the process of DENV
556 infection *in vivo* and the pathogenesis of dengue disease, although whether its
557 involvement is related to the virus entry stage or extends to the well-known participation
558 of this molecule in diverse signaling and regulation processes [49] remains to be
559 determined.

560 LRP1 is well conserved evolutionarily [49]. Its *A. aegypti* ortholog (NCBI Reference
561 Sequence: XP_021706493.1) exhibits 35% sequence identity with the human protein,
562 which is even higher (51%) across ligand-binding cluster IV, whose interaction with
563 DENV is strongest (Fig 3C, 4B). Were LRP1 to play a role in the entry of DENV into
564 mosquito cells similar to that described here in human cells, it would position this
565 receptor as a prime target for the development of antiviral strategies. However, in
566 contrast with our results, silencing of LRP1 orthologs in mosquito cells actually
567 increases the copy number of viral RNA in infected cells [28]. This paradoxical result,
568 according to the data published by Tree *et al*., stems from the involvement of LRP1 in
569 the regulation of intracellular cholesterol, whereby lower LRP1 levels would increase the
570 amount of intracellular cholesterol and, therefore, facilitate viral replication [28].
571 Undoubtedly, the precise role of LRP1 in DENV infection of human and mosquito cells
572 deserves the focus of further research efforts.

573 Our data prove that LRP1 binds DENV virions through protein E (Fig 3D and E), or
574 more specifically through DIII of protein E (Fig 4), although they do not rule out the
575 possible involvement of additional regions. DIII is the target of many highly potent

576 neutralizing antibodies [70–72] that have been shown to disrupt and block early
577 interaction events with cell surface molecules –presumably, the viral cell receptors [73].
578 This, together with the immunoglobulin-like fold of this domain and the capacity of
579 recombinant DIII to bind the cell surface [74] and compete with virus binding [6], has led
580 many to consider it a receptor-interacting region, despite the fact that the only receptors
581 DIII has been shown to bind are cell surface glycosaminoglycans [75]. The results
582 presented here indicate that the binding of protein E to LRP1 is conserved across all
583 four DENV serotypes, although there are considerable inter-serotype differences
584 regarding its affinity (Fig 4D).

585 It is also worth noting that several of the molecules described as DENV adhesion
586 receptors are members of well-known LRP1 functional complexes. For instance, many
587 LRP1 ligands use glycosaminoglycans as adhesion molecules [76], and proteins hsp90
588 [77], grp78 [78] and AXL [79], which have been separately identified as DENV adhesion
589 receptors [16,80,81], act cooperatively with LRP1 in binding, entry and signaling
590 processes.

591 LRP1 is ubiquitously expressed, including several tissues and cells types identified as
592 relevant for DENV replication such as monocytes/macrophages, keratinocytes, myeloid
593 cells, phagocytes, hepatocytes and endothelial cells [49,82]. Thus, we hypothesize that
594 LPR1 might constitute one of the hitherto unidentified endocytic receptors exploited by
595 DENV to gain entry into mammalian cells.

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600 **References**

601 1. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue:
602 a continuing global threat. Nat Rev Microbiol. 2010;8: S7–S16.
603 doi:10.1038/nrmicro2460

604 2. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The
605 global distribution and burden of dengue. Nature. 2013;496: 504–507.
606 doi:10.1038/nature12060

607 3. Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, et al.
608 Structure of dengue virus: implications for flavivirus organization, maturation, and
609 fusion. Cell. 2002;108: 717–725.

610 4. Rey FA. Dengue virus envelope glycoprotein structure: New insight into its
611 interactions during viral entry. Proc Natl Acad Sci. 2003;100: 6899–6901.
612 doi:10.1073/pnas.1332695100

613 5. Crill WD, Roehrig JT. Monoclonal Antibodies That Bind to Domain III of Dengue
614 Virus E Glycoprotein Are the Most Efficient Blockers of Virus Adsorption to Vero
615 Cells. J Virol. 2001;75: 7769–7773. doi:10.1128/JVI.75.16.7769-7773.2001

616 6. Chin JFL, Chu JJH, Ng ML. The envelope glycoprotein domain III of dengue virus
617 serotypes 1 and 2 inhibit virus entry. Microbes Infect. 2007;9: 1–6.
618 doi:10.1016/j.micinf.2006.09.009

619 7. Hung J-J, Hsieh M-T, Young M-J, Kao C-L, King C-C, Chang W. An external loop
620 region of domain III of dengue virus type 2 envelope protein is involved in serotype-
621 specific binding to mosquito but not mammalian cells. J Virol. 2004;78: 378–388.
622 doi:10.1128/jvi.78.1.378-388.2004

623 8. Chu JJH. Inhibition of West Nile virus entry by using a recombinant domain III from
624 the envelope glycoprotein. J Gen Virol. 2005;86: 405–412. doi:10.1099/vir.0.80411-
625 0

626 9. Krishnan MN, Sukumaran B, Pal U, Agaisse H, Murray JL, Hodge TW, et al. Rab 5
627 Is Required for the Cellular Entry of Dengue and West Nile Viruses. J Virol.
628 2007;81: 4881–4885. doi:10.1128/JVI.02210-06

629 10. Ayala-Nunez NV, Hoornweg TE, van de Pol DPI, Sjollema KA, Flipse J, van der
630 Schaar HM, et al. How antibodies alter the cell entry pathway of dengue virus
631 particles in macrophages. Sci Rep. 2016;6. doi:10.1038/srep28768

632 11. Moi ML, Lim C-K, Takasaki T, Kurane I. Involvement of the Fc receptor IIA
633 cytoplasmic domain in antibody-dependent enhancement of dengue virus infection.
634 J Gen Virol. 2010;91: 103–111. doi:10.1099/vir.0.014829-0

635 12. Rodrigo WWSI, Jin X, Blackley SD, Rose RC, Schlesinger JJ. Differential
636 Enhancement of Dengue Virus Immune Complex Infectivity Mediated by Signaling-
637 Competent and Signaling-Incompetent Human Fc RIA (CD64) or Fc RIIA (CD32). J
638 Virol. 2006;80: 10128–10138. doi:10.1128/JVI.00792-06

639 13. Rolph MS, Zaid A, Rulli NE, Mahalingam S. Downregulation of Interferon-β in
640 Antibody-Dependent Enhancement of Dengue Viral Infections of Human
641 Macrophages Is Dependent on Interleukin-6. J Infect Dis. 2011;204: 489–491.
642 doi:10.1093/infdis/jir271

643 14. Ubol S, Phuklia W, Kalayanarooj S, Modhiran N. Mechanisms of Immune Evasion
644 Induced by a Complex of Dengue Virus and Preexisting Enhancing Antibodies. J
645 Infect Dis. 2010;201: 923–935. doi:10.1086/651018

646 15. Li Y, Kakinami C, Li Q, Yang B, Li H. Human Apolipoprotein A-I Is Associated with
647 Dengue Virus and Enhances Virus Infection through SR-BI. Qiu J, editor. PLoS
648 ONE. 2013;8: e70390. doi:10.1371/journal.pone.0070390

649 16. Meertens L, Carnec X, Lecoin MP, Ramdasi R, Guivel-Benhassine F, Lew E, et al.
650 The TIM and TAM Families of Phosphatidylserine Receptors Mediate Dengue
651 Virus Entry. Cell Host Microbe. 2012;12: 544–557. doi:10.1016/j.chom.2012.08.009

652 17. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, et al. Dengue
653 virus infectivity depends on envelope protein binding to target cell heparan sulfate.
654 Nat Med. 1997;3: 866–871. doi:10.1038/nm0897-866

655 18. Navarro-Sanchez E, Altmeyer R, Amara A, Schwartz O, Fieschi F, Virelizier J, et al.
656 Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive
657 infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO
658 Rep. 2003;4: 723–728. doi:10.1038/sj.embor.embor866

659 19. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, Trumpfheller C, Finke J, Sun
660 W, et al. DC-SIGN (CD209) Mediates Dengue Virus Infection of Human Dendritic
661 Cells. J Exp Med. 2003;197: 823–829. doi:10.1084/jem.20021840

662 20. Dejnirattisai W, Webb AI, Chan V, Jumnainsong A, Davidson A, Mongkolsapaya J,
663 et al. Lectin Switching During Dengue Virus Infection. J Infect Dis. 2011;203: 1775–
664 1783. doi:10.1093/infdis/jir173

665 21. Miller JL, deWet BJM, Martinez-Pomares L, Radcliffe CM, Dwek RA, Rudd PM, et
666 al. The Mannose Receptor Mediates Dengue Virus Infection of Macrophages.
667 PLoS Pathog. 2008;4. doi:10.1371/journal.ppat.0040017

668 22. Lo Y-L, Liou G-G, Lyu J-H, Hsiao M, Hsu T-L, Wong C-H. Dengue Virus Infection Is
669 through a Cooperative Interaction between a Mannose Receptor and CLEC5A on
670 Macrophage as a Multivalent Hetero-Complex. Pöhlmann S, editor. PLOS ONE.
671 2016;11: e0166474. doi:10.1371/journal.pone.0166474

672 23. van der Schaar HM, Rust MJ, Chen C, van der Ende-Metselaar H, Wilschut J,
673 Zhuang X, et al. Dissecting the Cell Entry Pathway of Dengue Virus by Single-
674 Particle Tracking in Living Cells. Farzan M, editor. PLoS Pathog. 2008;4:
675 e1000244. doi:10.1371/journal.ppat.1000244

676 24. Dejarnac O, Hafirassou ML, Chazal M, Versapuech M, Gaillard J, Perera-Lecoin M,
677 et al. TIM-1 Ubiquitination Mediates Dengue Virus Entry. Cell Rep. 2018;23: 1779–
678 1793. doi:10.1016/j.celrep.2018.04.013

679 25. Lee C-J, Lin H-R, Liao C-L, Lin Y-L. Cholesterol Effectively Blocks Entry of
680 Flavivirus. J Virol. 2008;82: 6470–6480. doi:10.1128/JVI.00117-08

681 26. Biswas HH, Gordon A, Nuñez A, Perez MA, Balmaseda A, Harris E. Lower Low-
682 Density Lipoprotein Cholesterol Levels Are Associated with Severe Dengue
683 Outcome. Halstead SB, editor. PLoS Negl Trop Dis. 2015;9: e0003904.
684 doi:10.1371/journal.pntd.0003904

685 27. Melo CFOR, Delafiori J, Dabaja MZ, de Oliveira DN, Guerreiro TM, Colombo TE, et
686 al. The role of lipids in the inception, maintenance and complications of dengue
687 virus infection. Sci Rep. 2018;8: 11826. doi:10.1038/s41598-018-30385-x

688 28. Tree MO, Londono-Renteria B, Troupin A, Clark KM, Colpitts TM, Conway MJ.
689 Dengue virus reduces expression of low-density lipoprotein receptor-related protein
690 1 to facilitate replication in Aedes aegypti. Sci Rep. 2019;9: 6352.
691 doi:10.1038/s41598-019-42803-9

692 29. Guevara J, Romo J, McWhorter T, Guevara NV. Analogs of LDL Receptor Ligand
693 Motifs in Dengue Envelope and Capsid Proteins as Potential Codes for Cell Entry.
694 J Viruses. 2015;2015: 1–15. doi:10.1155/2015/646303

695 30. Holtzman DM, Herz J, Bu G. Apolipoprotein E and Apolipoprotein E Receptors:
696 Normal Biology and Roles in Alzheimer Disease. Cold Spring Harb Perspect Med.
697 2012;2. doi:10.1101/cshperspect.a006312

698 31. Chien Y-J, Chen W-J, Hsu W-L, Chiou S-S. Bovine lactoferrin inhibits Japanese
699 encephalitis virus by binding to heparan sulfate and receptor for low density
700 lipoprotein. Virology. 2008;379: 143–151. doi:10.1016/j.virol.2008.06.017

701 32. Jo-Mei Chen, Yi-Chin Fan, Jen-Wei Lin, Yi-Ying Chen, Wei-Li Hsu, Shyan-Song
702 Chiou. Bovine Lactoferrin Inhibits Dengue Virus Infectivity by Interacting with
703 Heparan Sulfate, Low-Density Lipoprotein Receptor, and DC-SIGN. Int J Mol Sci.
704 2017;18: 1957. doi:10.3390/ijms18091957

705 33. Herz J. The LDL Receptor Gene Family. Neuron. 2001;29: 571–581.
706 doi:10.1016/S0896-6273(01)00234-3

707 34. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL Receptor-Related
708 Protein 1: Unique Tissue-Specific Functions Revealed by Selective Gene Knockout
709 Studies. Physiol Rev. 2008;88: 887–918. doi:10.1152/physrev.00033.2007

710 35. Ramos Y, Huerta V, Martín D, Palomares S, Yero A, Pupo D, et al. An “on-matrix”
711 digestion procedure for AP-MS experiments dissects the interplay between
712 complex-conserved and serotype-specific reactivities in Dengue virus-human
713 plasma interactome. J Proteomics. 2017 [cited 8 Nov 2017].
714 doi:10.1016/j.jprot.2017.07.004

715 36. Huerta V, Ramos Y, Yero A, Pupo D, Martín D, Toledo P, et al. Novel interactions
716 of domain III from the envelope glycoprotein of dengue 2 virus with human plasma
717 proteins. J Proteomics. 2016;131: 205–213. doi:10.1016/j.jprot.2015.11.003

718 37. Huerta V, Toledo P, Fleitas N, Martín A, Pupo D, Yero A, et al. Receptor-activated
719 human α2-macroglobulin interacts with the envelope protein of dengue virus and
720 protects virions from temperature-induced inactivation through multivalent binding.
721 J Gen Virol. 2014;95: 2668–2676. doi:10.1099/vir.0.068544-0

722 38. Huerta V, Ramos Y, Yero A, Pupo D, Martin D, Márquez G, et al. Dataset on
723 protein composition of a human plasma sub-proteome able to modulate the
724 Dengue 2 virus infection in Huh 7.5 cells. Data Brief. 2016;6: 352–358.
725 doi:10.1016/j.dib.2015.12.016

726 39. Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of
727 Bacteriophage T4. Nature. 1970;227: 680–685. doi:10.1038/227680a0

728 40. Heukeshoven J, Dernick R. Simplified method for silver staining of proteins in
729 polyacrylamide gels and the mechanism of silver staining. Electrophoresis. 1985;6:
730 103–112. doi:10.1002/elps.1150060302

731 41. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from
732 polyacrylamide gels to nitrocellulose sheets: procedure and some applications.
733 Proc Natl Acad Sci. 1979;76: 4350–4354. doi:10.1073/pnas.76.9.4350

734 42. de Winter JCF. Using the Student’s t-test with extremely small sample sizes. Pract
735 Assess Res Eval. 2013;18: 1–12.

736 43. Balsitis SJ, Coloma J, Castro G, Alava A, Flores D, McKerrow JH, et al. Tropism of
737 dengue virus in mice and humans defined by viral nonstructural protein 3-specific
738 immunostaining. Am J Trop Med Hyg. 2009;80: 416–424.

739 44. Laatsch A, Panteli M, Sornsakrin M, Hoffzimmer B, Grewal T, Heeren J. Low
740 Density Lipoprotein Receptor-Related Protein 1 Dependent Endosomal Trapping
741 and Recycling of Apolipoprotein E. PLoS ONE. 2012;7.
742 doi:10.1371/journal.pone.0029385

743 45. Zamule SM, Strom SC, Omiecinski CJ. Preservation of hepatic phenotype in
744 lentiviral-transduced primary human hepatocytes. Chem Biol Interact. 2008;173:
745 179–186. doi:10.1016/j.cbi.2008.03.015

746 46. Finkelshtein D, Werman A, Novick D, Barak S, Rubinstein M. LDL receptor and its
747 family members serve as the cellular receptors for vesicular stomatitis virus. Proc
748 Natl Acad Sci U S A. 2013;110: 7306–7311. doi:10.1073/pnas.1214441110

749 47. Strickland DK, Ashcom JD, Williams S, Burgess WH, Migliorini M, Argraves WS.
750 Sequence identity between the alpha 2-macroglobulin receptor and low density
751 lipoprotein receptor-related protein suggests that this molecule is a multifunctional
752 receptor. J Biol Chem. 1990;265: 17401–17404.

753 48. Quinn KA, Grimsley PG, Dai YP, Tapner M, Chesterman CN, Owensby DA.
754 Soluble low density lipoprotein receptor-related protein (LRP) circulates in human
755 plasma. J Biol Chem. 1997;272: 23946–23951. doi:10.1074/jbc.272.38.23946

756 49. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related
757 protein 1: unique tissue-specific functions revealed by selective gene knockout
758 studies. Physiol Rev. 2008;88: 887–918. doi:10.1152/physrev.00033.2007

759 50. Huerta V, Ramos Y, Yero A, Pupo D, Martin D, Márquez G, et al. Dataset on
760 protein composition of a human plasma sub-proteome able to modulate the
761 Dengue 2 virus infection in Huh 7.5 cells. Data Brief. 2016;6: 352–358.
762 doi:10.1016/j.dib.2015.12.016

763 51. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the
764 flavivirus life cycle. Nat Rev Microbiol. 2005;3: 13–22. doi:10.1038/nrmicro1067

765 52. Thullier P, Demangel C, Bedouelle H, Mégret F, Jouan A, Deubel V, et al. Mapping
766 of a dengue virus neutralizing epitope critical for the infectivity of all serotypes:
767 insight into the neutralization mechanism. J Gen Virol. 2001;82: 1885–1892.
768 doi:10.1099/0022-1317-82-8-1885

769 53. Chin JFL, Chu JJH, Ng ML. The envelope glycoprotein domain III of dengue virus
770 serotypes 1 and 2 inhibit virus entry. Microbes Infect. 2007;9: 1–6.
771 doi:10.1016/j.micinf.2006.09.009

772 54. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et
773 al. Clustal W and Clustal X version 2.0. Bioinforma Oxf Engl. 2007;23: 2947–2948.
774 doi:10.1093/bioinformatics/btm404

775 55. Robert X, Gouet P. Deciphering key features in protein structures with the new
776 ENDscript server. Nucleic Acids Res. 2014;42: W320-324. doi:10.1093/nar/gku316

777 56. Fisher C, Beglova N, Blacklow SC. Structure of an LDLR-RAP complex reveals a
778 general mode for ligand recognition by lipoprotein receptors. Mol Cell. 2006;22:
779 277–283. doi:10.1016/j.molcel.2006.02.021

780 57. Jensen JK, Dolmer K, Gettins PGW. Specificity of binding of the low density
781 lipoprotein receptor-related protein to different conformational states of the clade E
782 serpins plasminogen activator inhibitor-1 and proteinase nexin-1. J Biol Chem.
783 2009;284: 17989–17997. doi:10.1074/jbc.M109.009530

784 58. Dolmer K, Campos A, Gettins PGW. Quantitative dissection of the binding
785 contributions of ligand lysines of the receptor-associated protein (RAP) to the low
786 density lipoprotein receptor-related protein (LRP1). J Biol Chem. 2013;288: 24081–
787 24090. doi:10.1074/jbc.M113.473728

788 59. Williams SE, Inoue I, Tran H, Fry GL, Pladet MW, Iverius PH, et al. The carboxyl-
789 terminal domain of lipoprotein lipase binds to the low density lipoprotein receptor-
790 related protein/alpha 2-macroglobulin receptor (LRP) and mediates binding of
791 normal very low density lipoproteins to LRP. J Biol Chem. 1994;269: 8653–8658.

792 60. Jensen GA, Andersen OM, Bonvin AMJJ, Bjerrum-Bohr I, Etzerodt M, Thøgersen
793 HC, et al. Binding site structure of one LRP-RAP complex: implications for a
794 common ligand-receptor binding motif. J Mol Biol. 2006;362: 700–716.
795 doi:10.1016/j.jmb.2006.07.013

796 61. van den Biggelaar M, Madsen JJ, Faber JH, Zuurveld MG, van der Zwaan C,
797 Olsen OH, et al. Factor VIII Interacts with the Endocytic Receptor Low-density
798 Lipoprotein Receptor-related Protein 1 via an Extended Surface Comprising “Hot-
799 Spot” Lysine Residues. J Biol Chem. 2015;290: 16463–16476.
800 doi:10.1074/jbc.M115.650911

801 62. Boulant S, Stanifer M, Lozach P-Y. Dynamics of Virus-Receptor Interactions in
802 Virus Binding, Signaling, and Endocytosis. Viruses. 2015;7: 2794–2815.
803 doi:10.3390/v7062747

804 63. Noisakran S, Onlamoon N, Songprakhon P, Hsiao H-M, Chokephaibulkit K, Perng
805 GC. Cells in Dengue Virus Infection In Vivo. Adv Virol. 2010;2010: 1–15.
806 doi:10.1155/2010/164878

807 64. Perera-Lecoin M, Meertens L, Carnec X, Amara A. Flavivirus Entry Receptors: An
808 Update. Viruses. 2013;6: 69–88. doi:10.3390/v6010069

809 65. Huerta V, Toledo P, Fleitas N, Martín A, Pupo D, Yero A, et al. Receptor-activated
810 human α2-macroglobulin interacts with the envelope protein of dengue virus and
811 protects virions from temperature-induced inactivation through multivalent binding.
812 J Gen Virol. 2014;95: 2668–2676. doi:10.1099/vir.0.068544-0

813 66. Ramos Y, Huerta V, Martín D, Palomares S, Yero A, Pupo D, et al. An “on-matrix”
814 digestion procedure for AP-MS experiments dissects the interplay between
815 complex-conserved and serotype-specific reactivities in Dengue virus-human
816 plasma interactome. J Proteomics. 2017 [cited 11 Oct 2018].
817 doi:10.1016/j.jprot.2017.07.004

818 67. Krause K, Azouz F, Nakano E, Nerurkar VR, Kumar M. Deletion of Pregnancy
819 Zone Protein and Murinoglobulin-1 Restricts the Pathogenesis of West Nile Virus
820 Infection in Mice. Front Microbiol. 2019;10. doi:10.3389/fmicb.2019.00259

821 68. Pietrzik C, Jaeger S. Functional Role of Lipoprotein Receptors in Alzheimers
822 Disease. Curr Alzheimer Res. 2008;5: 15–25. doi:10.2174/156720508783884675

823 69. Nikolayeva I, Bost P, Casademont I, Duong V, Koeth F, Prot M, et al. A Blood RNA
824 Signature Detecting Severe Disease in Young Dengue Patients at Hospital Arrival.
825 J Infect Dis. 2018;217: 1690–1698. doi:10.1093/infdis/jiy086

826 70. Beltramello M, Williams KL, Simmons CP, Macagno A, Simonelli L, Quyen NTH, et
827 al. The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-
828 Reactive Antibodies Endowed with Neutralizing and Enhancing Activity. Cell Host
829 Microbe. 2010;8: 271–283. doi:10.1016/j.chom.2010.08.007

830 71. Wahala WMPB, de Silva AM. The Human Antibody Response to Dengue Virus
831 Infection. Viruses. 2011;3: 2374–2395. doi:10.3390/v3122374

832 72. Gromowski GD, Barrett ND, Barrett ADT. Characterization of Dengue Virus
833 Complex-Specific Neutralizing Epitopes on Envelope Protein Domain III of Dengue
834 2 Virus. J Virol. 2008;82: 8828–8837. doi:10.1128/JVI.00606-08

835 73. Crill WD, Roehrig JT. Monoclonal Antibodies That Bind to Domain III of Dengue
836 Virus E Glycoprotein Are the Most Efficient Blockers of Virus Adsorption to Vero
837 Cells. J Virol. 2001;75: 7769–7773. doi:10.1128/JVI.75.16.7769-7773.2001

838 74. Hung J-J, Hsieh M-T, Young M-J, Kao C-L, King C-C, Chang W. An External Loop
839 Region of Domain III of Dengue Virus Type 2 Envelope Protein Is Involved in
840 Serotype-Specific Binding to Mosquito but Not Mammalian Cells. J Virol. 2004;78:
841 378–388. doi:10.1128/JVI.78.1.378-388.2004

842 75. Watterson D, Kobe B, Young PR. Residues in domain III of the dengue virus
843 envelope glycoprotein involved in cell-surface glycosaminoglycan binding. J Gen
844 Virol. 2012;93: 72–82. doi:10.1099/vir.0.037317-0

845 76. Kanekiyo T, Zhang J, Liu Q, Liu C-C, Zhang L, Bu G. Heparan Sulphate
846 Proteoglycan and the Low-Density Lipoprotein Receptor-Related Protein 1
847 Constitute Major Pathways for Neuronal Amyloid-β Uptake. J Neurosci. 2011;31:
848 1644–1651. doi:10.1523/JNEUROSCI.5491-10.2011

849 77. Gopal U, Bohonowych JE, Lema-Tome C, Liu A, Garrett-Mayer E, Wang B, et al. A
850 Novel Extracellular Hsp90 Mediated Co-Receptor Function for LRP1 Regulates
851 EphA2 Dependent Glioblastoma Cell Invasion. PLOS ONE. 2011;6: e17649.
852 doi:10.1371/journal.pone.0017649

853 78. Kant Misra U, Gonzalez-Gronow M, Gawdi G, Wang F, Vincent Pizzo S. A novel
854 receptor function for the heat shock protein Grp78: silencing of Grp78 gene
855 expression attenuates α2M*-induced signalling. Cell Signal. 2004;16: 929–938.
856 doi:10.1016/j.cellsig.2004.01.003

857 79. Subramanian M, Hayes CD, Thome JJ, Thorp E, Matsushima GK, Herz J, et al. An
858 AXL/LRP-1/RANBP9 complex mediates DC efferocytosis and antigen cross-
859 presentation in vivo. J Clin Invest. 2014;124: 1296–1308. doi:10.1172/JCI72051

860 80. Jindadamrongwech S, Thepparit C, Smith DR. Identification of GRP 78 (BiP) as a
861 liver cell expressed receptor element for dengue virus serotype 2. Arch Virol.
862 2004;149: 915–927. doi:10.1007/s00705-003-0263-x

863 81. Reyes-del Valle J, Chavez-Salinas S, Medina F, del Angel RM. Heat Shock Protein
864 90 and Heat Shock Protein 70 Are Components of Dengue Virus Receptor
865 Complex in Human Cells. J Virol. 2005;79: 4557–4567. doi:10.1128/JVI.79.8.4557-
866 4567.2005

867 82. Moestrup SK, Gliemann J, Pallesen G. Distribution of the alpha 2-macroglobulin
868 receptor/low density lipoprotein receptor-related protein in human tissues. Cell
869 Tissue Res. 1992;269: 375–382. doi:10.1007/bf00353892

870

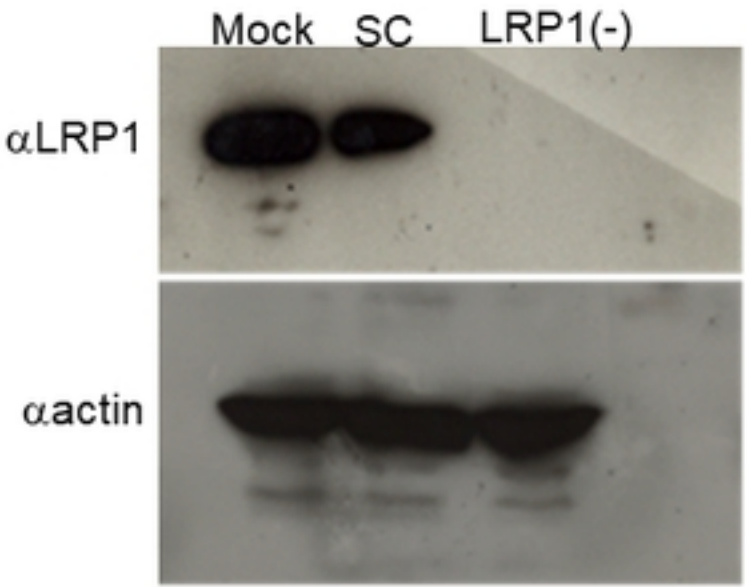
871 ## Supporting information S1

872 **Table A. Factor of enrichment of LRP1 proteotypic peptides respect to the**
873 **background control in the Pull Down experiments.** [1]P-value calculated for the T-test
874 analysis of each experimental condition means (DIIIE1, DIIIE2, DIIIE3 or DIIIE4) versus
875 the background means of normalized intensity values. (*), denotes P-value below 0.05,
876 (ns) no significative. The Skyline software was used to carry out the statistical analysis.

877 **Table B. Isoelectric point and charge calculations**.

878 **Table C. Sequence identity* and similarity of DIII from DENV1-4**. *values located
879 over (under) the diagonal correspond to sequence identity (similarity).

(A)


Mock
SC
LRP1(-)
αLRP1
αactin

(B)

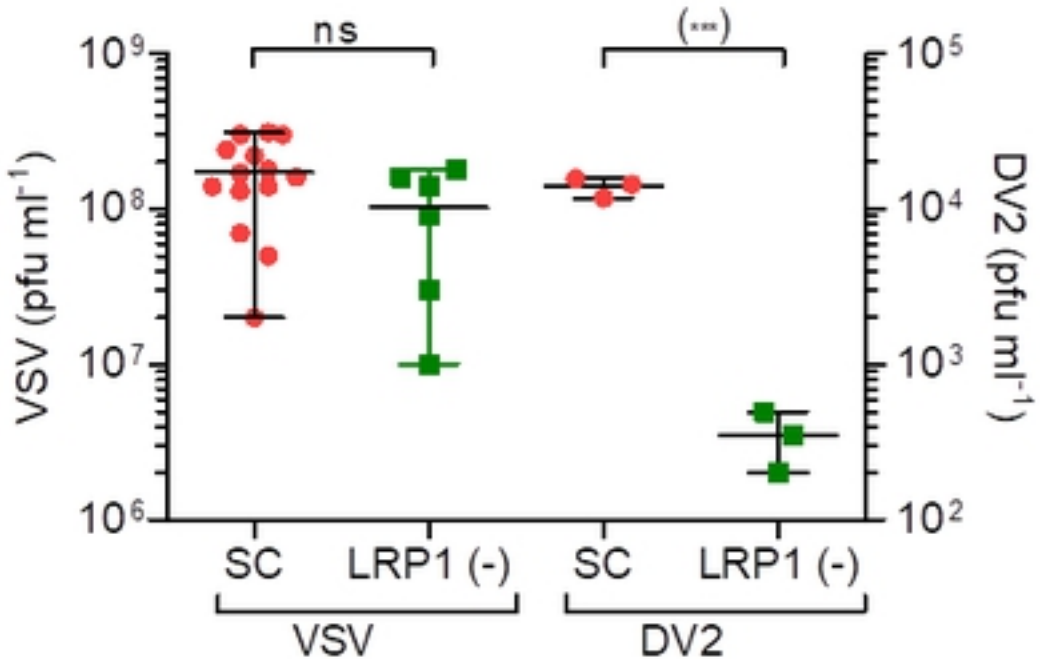

ns
(***)
VSV (pfu ml-1)
DV2 (pfu ml-1)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
SC
LRP1 (-)
SC
LRP1 (-)
VSV
DV2

Fig. 1

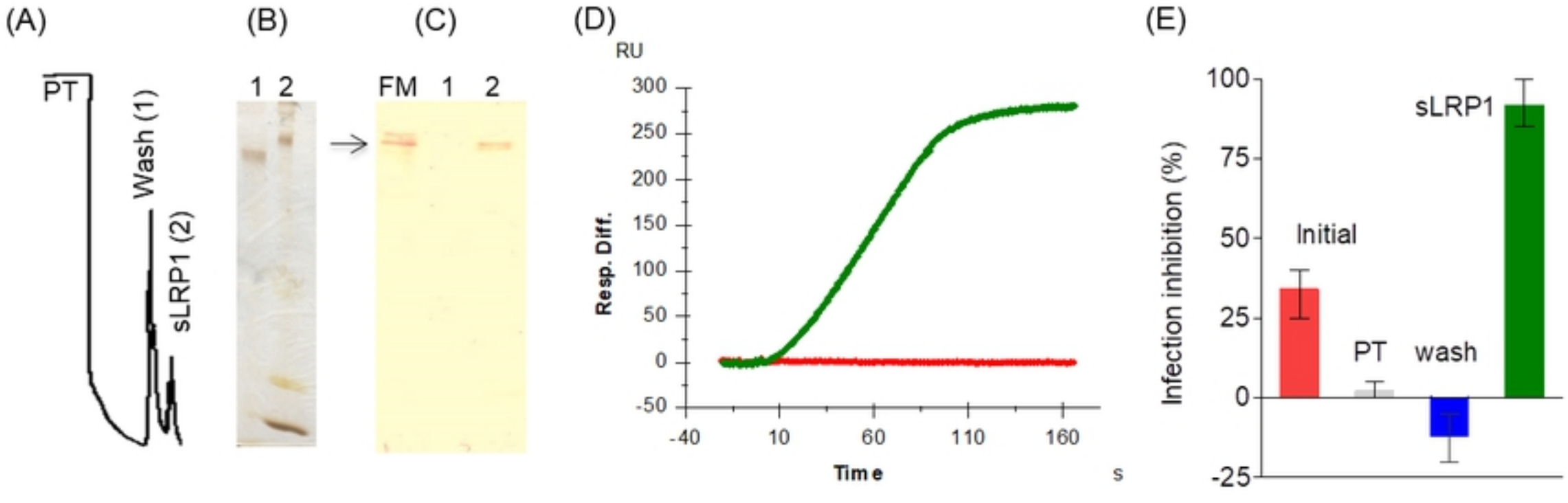

(A)
PT
Wash (1)
sLRP1 (2)
(B)
1 2
(C)
FM 1 2
(D)
RU
300
250
200
150
100
50
0
-50
Resp. Diff.
-40
10
60
110
160
Time
s
(E)
100
75
50
25
0
-25
Infection inhibition (%)
Initial
PT
wash
sLRP1

Fig. 2

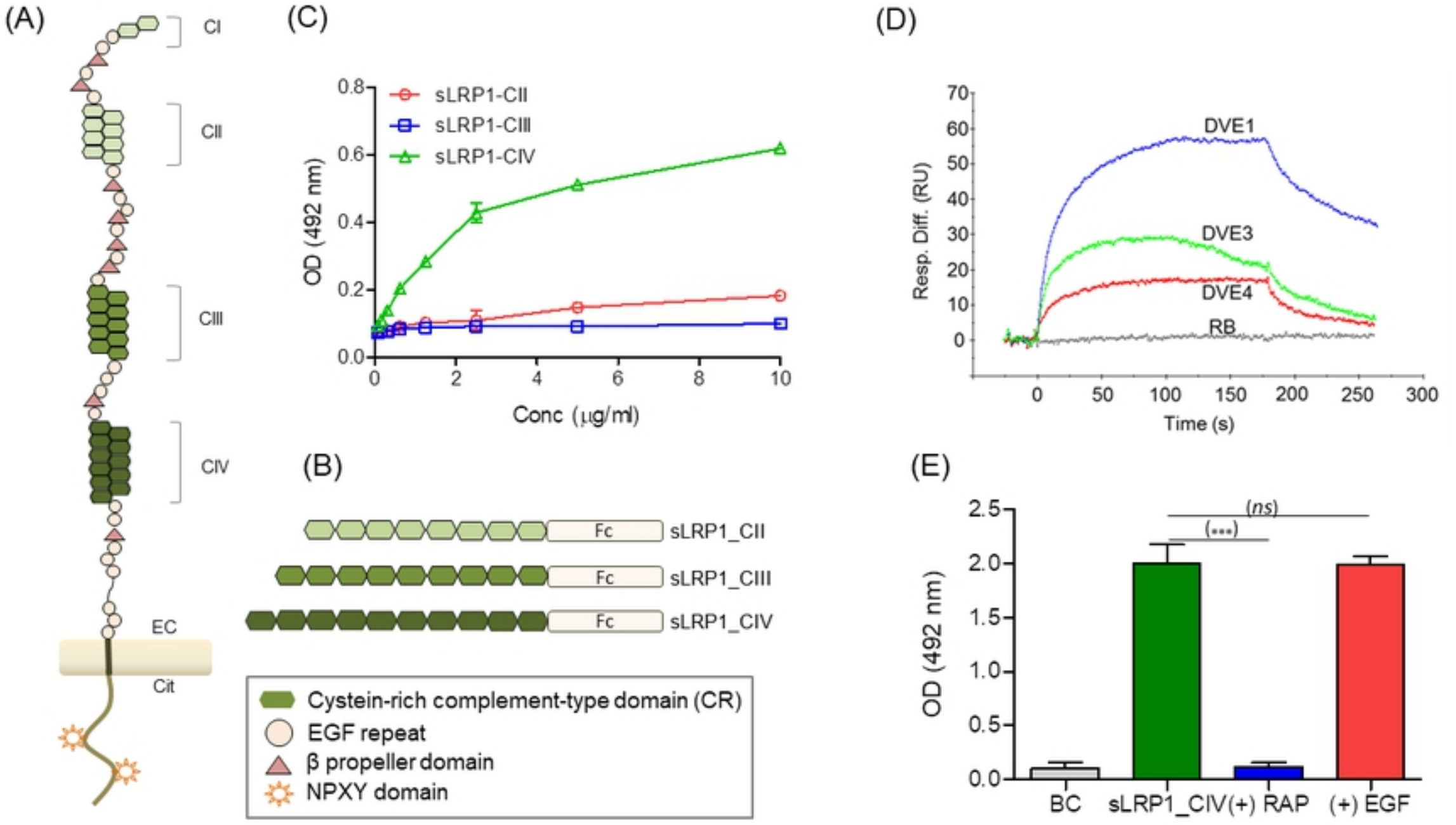

(A)
CI
CII
CIII
CIV
EC
Cit
(C)
sLRP1-CII
sLRP1-CIII
sLRP1-CIV
OD (492 nm)
Conc (µg/ml)
(D)
DVE1
DVE3
DVE4
RB
Resp. Diff. (RU)
Time (s)
(B)
Fc
sLRP1_CII
sLRP1_CIII
sLRP1_CIV
Cystein-rich complement-type domain (CR)
EGF repeat
β propeller domain
NPXY domain
(E)
(ns)
(***)
OD (492 nm)
BC
sLRP1_CIV
(+) RAP
(+) EGF

Fig. 3

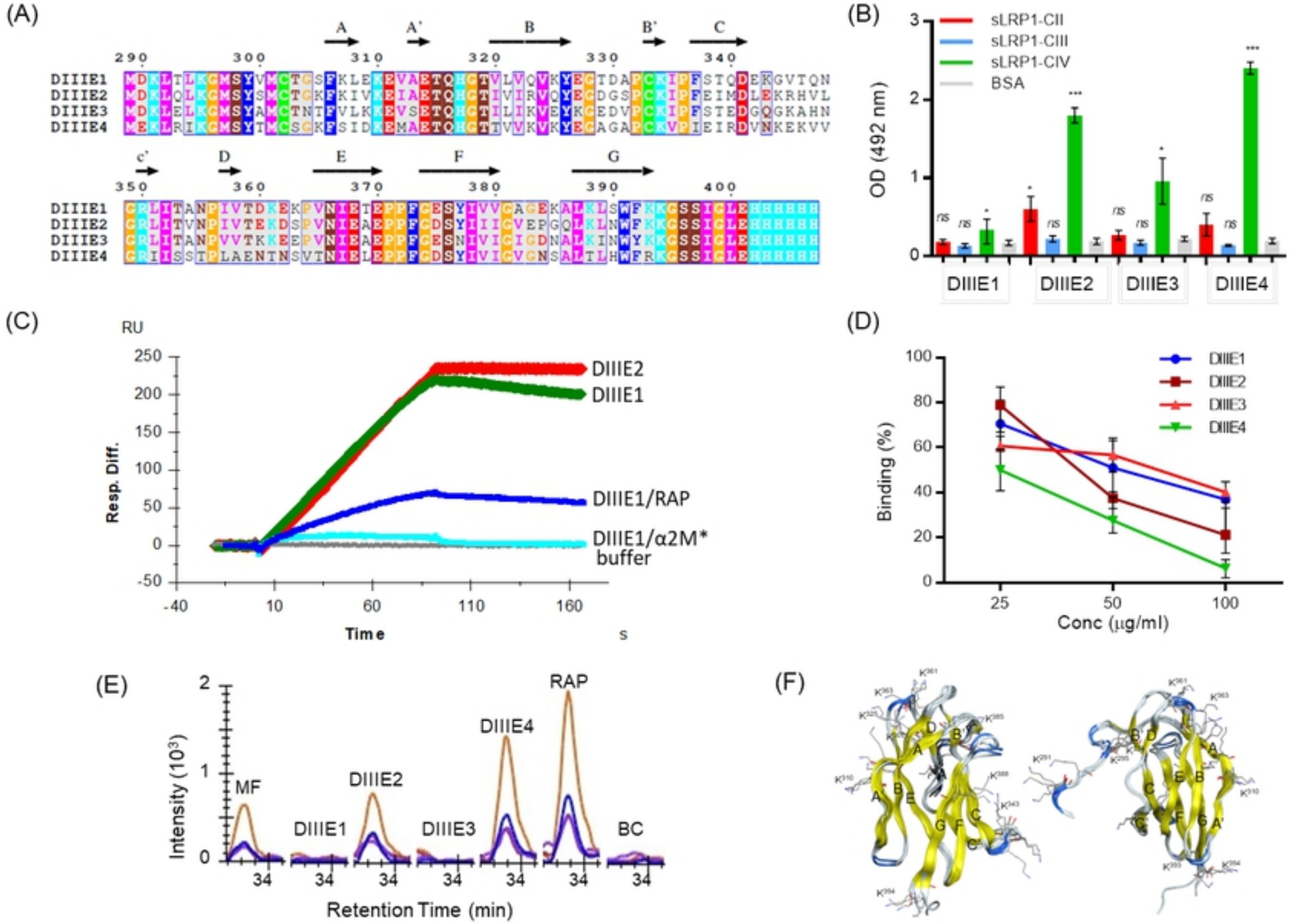

(A)
A
A'
B
B'
C
290
300
310
320
330
340
DIIIE1
DIIIE2
DIIIE3
DIIIE4
c'
D
E
F
G
350
360
370
380
390
400
(B)
sLRP1-CII
sLRP1-CIII
sLRP1-CIV
BSA
OD (492 nm)
ns
DIIIE1
DIIIE2
DIIIE3
DIIIE4
(C)
RU
Resp. Diff.
DIIIE2
DIIIE1
DIIIE1/RAP
DIIIE1/α2M*
buffer
Time
s
(D)
DIIIE1
DIIIE2
DIIIE3
DIIIE4
Binding (%)
Conc (μg/ml)
(E)
Intensity (10³)
MF
DIIIE1
DIIIE2
DIIIE3
DIIIE4
RAP
BC
Retention Time (min)
(F)

Fig. 4