The dengue virus non-structural protein 1 (NS1) use the scavenger receptor B1 as cell receptor in human hepatic and mosquito cells.

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Abstract

Dengue is the most common virus disease transmitted to humans by mosquitoes. The dengue virus NS1 is a multifunctional protein that form part of replication complexes. In addition, NS1 is also secreted, as a hexamer, to the extracellular milieu. Circulating NS1 has been associated with dengue pathogenesis by several different mechanisms. Cell binding and internalization of soluble NS1 result in the disruption of tight junctions and in down regulation of the innate immune response. In this work, we report that the HDL scavenger receptor B1 (SRB1) in human hepatic cells, and a scavenger receptor B1-like in mosquito C6/36 cells act as cell surface binding receptor for dengue virus NS1. The presence of the SRB1 on the plasma membrane of C6/36 cells, as well as in Huh-7 cells, was demonstrated by confocal microscopy. Internalization of NS1 can be efficiently blocked by anti-SRB1 antibodies and previous incubation of the cells with HDL significantly reduces NS1 internalization. In addition, the transient expression of SRB1 in Vero cells, which lack the receptor, renders these cells susceptible to NS1 entry. Direct interaction between soluble NS1 and the SRB1 in Huh7 and C6/36 cells was demonstrated in vivo by proximity ligation assays and in vitro by surface plasmon resonance. Finally, data is presented indicating that the SRB1 also act as cell receptor for zika virus NS1. These results demonstrate that dengue virus NS1, a *bona fide* lipoprotein, usurps the HDL receptor for cell entry and offers explanations for the altered serum lipoprotein homeostasis observed in dengue patients.

Key words: dengue, dengue virus, zika virus, NS1, scavenger receptor B-1.

39 Introduction

40 The *Flavivirus* genus, belonging to the *Flaviviridae* family, includes a group of
 41 vector borne viruses of importance in human public health as dengue virus
 42 (DENV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus
 43 (JEV) and West Nile virus (WNV). DENV and ZIKV are transmitted by the same
 44 arthropods of the *Aedes* genus and share a similar geographical distribution
 45 (Paixão et al 2018). It is estimated that almost half of the world population,
 46 distributed in tropical and subtropical zones, is at risk of contracting these
 47 diseases (WHO, 2018). The initial symptoms of both DENV and ZIKV infections
 48 manifest as a febrile syndrome. Infections with DENV can evolve to
 49 hemorrhagic syndrome accompanied of hypovolemic shock, systemic failure
 50 and death, mainly in children. In turn, ZIKV infections when acquired during
 51 pregnancy, are associated with the occurrence of microcephaly and other
 52 neurological severe injuries in the fetus brain (Rather et al 2017). In adults,
 53 ZIKV has been associated with the occurrence of Guillan-Barré syndrome which
 54 is highly disabling (Song et al., 2017). DENV and ZIKV virions are spherical
 55 particles of about 50nm in diameter. The positive RNA genome is about 11Kb in
 56 length encoding for 3 structural (Membrane, Capsid and Envelope) and for 7
 57 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The
 58 gene encoding for the NS1 is 1056 ntds in length rendering a 48-54 KDa protein
 59 depending on its glycosylation status. The NS1 could be found in monomeric,
 60 dimeric or hexameric forms (Muller and Young 2013). Structurally, the
 61 monomeric form of NS1 is composed of three domains: α/β wing (RIG-I-like
 62 folded, from amino acids 38–151), β -roll (hydrophobic, from amino acids 1–29)
 63 and β -ladder (composed of a β -sheet and a spaghetti loop, from aminoacids
 64 181–352) (Akey et al., 2015). Once the NS1 is translated from the viral genome,
 65 it is located at the lumen of the ER in a monomeric form which rapidly dimerizes
 66 (reviewed in Muller and Young, 2013). The intracellular NS1 is mainly dimeric
 67 and is associated to intra and extracellular membranes participating in different
 68 processes interacting with cellular and viral proteins during the viral replication,
 69 virion assembly, signaling transduction and evasion of the cellular immune
 70 response (Jacobs et al., 2000; Scaturro et al., 2015). The hexameric form of
 71 NS1 is an association of three dimers assembled in a hollow barrel-shaped
 72 structure, with a central channel rich in lipids acquired from cellular membranes
 73 (Gutshe et al., 2011). NS1 is the only flaviviral nonstructural protein secreted
 74 together with the viral particles toward the extracellular milieu from infected
 75 vertebrate and insect cells in high concentrations (up to $\mu\text{g/mL}$) (Flamand et al.,
 76 1999; Alcalá et al., 2016). In fact, NS1 is used as an early marker for DENV and
 77 ZIKV detection from patient serum samples (reviewed in De la Cruz Hernández
 78 et al., 2013). The DENV, soluble circulating NS1 has been involved in several
 79 pathogenic mechanisms during the infection process. It has been reported NS1
 80 can affect the complement pathway promoting the degradation of C4 protein,
 81 alters the coagulation system inhibiting the activation of prothrombin (reviewed

82 by Conde et al., 2017). NS1 can also disrupts endothelium integrity, promoting
83 an increase in the vascular permeability after internalization by endocytosis, that
84 may be related to plasma leakage (Glasner et al., 2017; Puerta-Guardo et al.,
85 2019; Wang et al., 2019). Moreover, pre-exposure of human liver or dendritic
86 cells to NS1 renders these cells more susceptible to DENV replication (Alcon-
87 LePoder et al, 2005; Alayli and Scholle, 2016). Thus, a better understanding of
88 the DENV soluble NS1- cell interactions may result in strategies to combat
89 DENV replication and pathogenesis.

90 The scavenger receptor B type 1 (SRB1) or his human homolog CLA-1, is a 509
91 amino acid transmembrane glycoprotein containing an extracellular domain as a
92 unique loop of 408 amino acids with multiple sites of N-glycosylation and a
93 cysteine-rich region, 2 transmembrane domains of 22 and 23 amino acids and 2
94 cytoplasmatic N and C terminal domains of 9 and 44 amino acids, respectively
95 (Shen et al, 2018). SRB1 is the physiologically relevant cell surface HDL
96 receptor responsible for selective HDL-cholesteryl esters (HDL-CE) uptake
97 mainly in liver, where it is most abundantly expressed, providing cholesterol for
98 bile acid synthesis and controlling de plasma HDL levels (Acton et al., 1996;
99 Shen, 2019). In insects as *Drosophila melanogaster* and *Aedes aegypti*, among
100 others, homolog genes for SRB1 have been identified; however, their functions
101 in fatty acid transport has not been studied in these organisms (Nichols et al.,
102 2008).

103 The SRB1 has been described as the principal receptor of hepatitis C virus
104 (HCV) in hepatocytes, through the recognition of the E2 hypervariable region 1
105 of the HCV envelope lipoprotein (Catanese et al., 2010). In addition, it has been
106 reported that apolipoprotein AI (ApoA-I) the principal carrier of HDL molecules,
107 bridges DENV particles and cell receptor SRB1 and facilitates entry of DENV
108 into cells enhancing the infection (Li et al., 2013). DENV has a broad tropism for
109 liver tissue where it replicates efficiently in hepatocytes; however, the elements
110 that direct this tropism are not known (Povoa et al., 2014). DENV NS1 can entry
111 into liver tissue cells both *in vitro* and *in vivo* in the absence of viral particles. In
112 addition, an increase in virus yield is observed from hepatic cells preincubated
113 with DENV NS1, and it has been postulated that DENV NS1 conditions the
114 hepatic cells to increase viral particle production (Alcon-Le Poder et al., 2005).
115 Recently, it was reported that endocytosis of NS1 is required for NS1-mediated
116 endothelial hyperpermeability (Wang et al., 2019). However, the host factors
117 favoring NS1 binding and entry in hepatocytes or other cells are unknown.

118 Since NS1 must be internalized to exert its functions and the DENV NS1 is a
119 *bonna-fide* glyco-lipoprotein, with a lipid cargo composed of triglycerides,
120 cholesteryl esters and phospholipids that resembles in composition the plasma
121 lipoprotein HDL (Gutshel et al., 2011), this suggested to us that DENV NS1
122 could mimic or hijack some of the lipid metabolic pathways to enter the cell. In
123 this work, we present evidence demonstrating that the HDL SRB1 in human

124 hepatic cells, and an SRB1-like molecule in mosquito cells, is also used by
125 DENV and ZIKV soluble NS1 as cell receptor for cell binding and internalization.

126 **Material and Methods**

127 **Virus and cells.** Dengue 4 virus (kindly donated by Dr. Juan Salas, Instituto
128 Politécnico Nacional, México) was propagated in suckling mouse brains as
129 previously described (Gould and Cleg, 1991). Zika virus strain M7366 (kindly
130 donated by Dr. Susana López, Instituto de Biotecnología, UNAM, Mexico) was
131 propagated in C6/36 cells. Virus titers from mice brain homogenates or cell
132 culture supernatant were determined by plaque assay as described by Ludert et
133 al. (2008) but using Vero cells grown in the cells culture conditions described in
134 this work. C6/36 cells (ATCC, USA) were grown in EMEM medium at 28 °C and
135 5% CO₂ atmosphere. Huh-7 and Vero cells were grown in DMEM medium
136 (Sigma, USA) at 37 °C and 8% CO₂ atmosphere. All cell culture media were
137 supplemented with 5% FBS.

138 **Cell surface localization of SBR1.** Confluent monolayers grown in LAB-TEK®
139 (Nalgene Nunc International, USA) were fixed (4% paraformaldehyde-PBS) for
140 10 min at RT and then incubated with blocking buffer (10% FBS, 3%BSA,
141 100mM Glycine in PBS) for 45 min at RT. The primary antibodies anti-SRB1
142 rabbit Mab (Abcam, USA) was diluted 1:300 (10% FBS, 1% BSA, 100mM
143 Glycine in PBS) and incubated with the cells for 1h, 37°C. After 5 washes, cells
144 were incubated with an anti-rabbit Alexa Fluor 594 (Invitrogen, USA) diluted
145 1:1000 in PBS. After 10 washes, the cells were incubated with Dapi (4',6-
146 Diamidine-2'-phenylindole dihydrochloride) in mounting medium (Molecular
147 Probes). Afterward, a coverslip was added and sealed with nail polish. The
148 immunolabeled cells were observed in the inverted confocal microscope
149 (Olympus MPhot) and the images were analyzed in the Image J/FIJI software.
150 In order to capture images with higher resolution of the molecules located on
151 the plasma membranes, monolayers of Huh-7 and C6/36 were cells grown in
152 Fluorodish Cell Cultures (WPI, USA) and the SRB-1 coimmunolabelled with
153 wheat germ agglutinin conjugated with Alexa Fluor488 (Thermofisher, USA) to
154 observed the silhouette of the cells and the integrity of the plasmatic membrane.
155 Colabelled cells were examined by total internal reflection fluorescence
156 microscopy (TIRF) (Olympus IX81 TIRF). The antibodies and conditions used in
157 TIRF were as described for the confocal microscopy.

158 **NS1 internalization assays.** Huh-7 and C6/36 cells were grown in 8 well
159 chambers (LAB-TEK®, Nalgene Nunc International, USA) for 24h, until confluent
160 monolayers were formed. Monolayers were incubated with 3,5 µg/well of
161 recombinant DENV or ZIKV NS1 protein (Aalto Bio Reagents, Dublin). After
162 1,5h incubation, at the cell specific growth temperature, cells were washed
163 extensively, fixed with 4% paraformaldehyde-PBS for 10 min at RT and
164 permeabilized with 0,1 % Triton-PBS, for 5 min at RT and finally incubated with

165 blocking buffer (10% FBS, 3% BSA, 100mM Glycine in PBS) for 45 min at RT.
 166 Internalized NS1 was revealed by immunolabeling using an anti-NS1 Mab
 167 (kindly donated by Prof. Eva Harris, University of California, Berkeley) diluted
 168 1:300 (10% FBS, 1% BSA, 100mM Glycine in PBS) as primary antibody
 169 incubated for 1h at 37°C. After 5 washes, cells were incubated with an anti-
 170 mouse Alexa fluor 568 (Invitrogen, USA) diluted 1:1000 in PBS as secondary
 171 antibody. After 10 washes, the cells were incubated with Dapi (4',6-Diamidine-
 172 2'-phenylindole dihydrochloride) in mounting medium (Molecular Probes).
 173 Afterward, a coverslip was added and sealed with nail polish. The
 174 immunolabeled cells were observed in an inverted confocal microscope
 175 (Olympus MPhot) and images analyzed with Image J/FIJI software. For the
 176 quantification of NS1 levels inside the cells, 10 cells by field on 3 fields by
 177 condition, were selected as Region of Interest (ROI), and arbitrary fluorescent
 178 units were measured using the Image J/Fiji software. The means for each
 179 condition were calculated and the analysis of variance (ANOVA) one-tailed test
 180 used to compare the mean of fluorescent arbitrary units (FAU) from each
 181 measure.

182 **DENV and ZIKV infection in insect cells preincubated with recombinant**
 183 **NS1.** C6/36 cell monolayers grown in 24-well plates were preincubated or not
 184 with 3,5 and 7,0 µg/well of recombinant DENV or ZIKV NS1 protein for 1,5 h.
 185 After extensive washing, cells were inoculated with DENV or ZIKV at a MOI=1.
 186 Infection was left to proceed for 48 hours, the supernatants collected, and the
 187 virus yield titrated by plaque assay using Vero cells, as previously described
 188 (Ludert et al., 2008).

189 **Inhibition of NS1 entry by preincubation with anti-SRB1 antibodies.**
 190 Confluent monolayers of Huh-7 and C6/36 cells, grown in 8-well chambers LAB-
 191 TEK® (Nalgene Nunc International, USA) for 24h, were preincubated or not with
 192 anti-SRB1 antibody (Abcam) at concentrations of 1 and 10 µg/ml for 1h at 37°C.
 193 After extensive washing, cells were incubated with recombinant DENV NS1
 194 protein at a concentration of 3,5 µg/well for 1,5 h, and the cells fixed and
 195 processed for detection and quantification of internalized NS1 as described
 196 above.

197 **NS1 and HDL competition assays.** C6/36 or Huh7 were seeded in 8-well
 198 chambers and confluent monolayers were incubated with 150 or 300 µg/mL of
 199 HDL (Merck, USA) in specific growth medium without FBS for 1.5 h, at the
 200 appropriate cell growth conditions. After extensive washing, 3,5 µg/well of
 201 DENV or ZIKV recombinant NS1 protein were added in growth medium without
 202 FBS. After incubation for 1,5h at the cell specific growth conditions, cells were
 203 fixed, permeabilized and immunolabeled for detection of internalized DENV or
 204 ZIKV NS1 as describe above.

205 **Overexpression of SRB1 in Vero cells.** Monolayers, 80-90% of confluence, of
206 Vero cells seeded in 8-well chambers, were transfected with the plasmid pCMV-
207 SCARB1-His (Sinobiological, USA) for the expression of the SRB1.
208 Transfections were carried with Lipofectamine 2000 LTX (Invitrogen) according
209 to manufacturer's instructions using 0.5 µg/well of the plasmid. Twenty-four hpt,
210 supernatant were removed and the cells incubated with 3,5 µg/mL of
211 recombinant DENV NS1 in DMEM medium without FBS for 1.5h at the cells
212 growth conditions. Cells were fixed, permeabilized and co-immunolabeled with
213 anti-NS1 and anti-SRB1 specific primary antibodies and revealed and visualized
214 by confocal microscopy as describe above.

215 **Proximity Ligation Assays:** Interactions between the SRB1 and NS1 proteins
216 from DENV and ZIKV in Huh-7 and C6/36 cells were detected using the
217 commercial kit Duolink (Sigma-Aldrich) based on *in situ* proximity ligation assay
218 (PLA) used following manufacturer's instructions. Briefly, C6/36 or Huh-7
219 monolayers were grown on 8-well chambers, preincubated with DENV or ZIKV
220 NS1 recombinant proteins (3,5 µg/well), and after 45 min of incubation, fixed
221 with 4% paraformaldehyde for 10 min at RT. After incubation with blocking
222 buffer for 30 min at 37°C, cells were incubated with mouse anti-NS1 mAb
223 and rabbit anti-SRB1 polyclonal antibody (Abcam, USA) as primary antibodies.
224 After several washes, cells were incubated with the PLA PROBES linked, anti-
225 mouse and anti-rabbit secondary antibodies. Finally, the cells were subjected to
226 ligation and amplification reactions. The specificity of PLA was evaluated using
227 recombinant NS1 exposed cells incubated only with the NS1 specific antibody
228 and the two PLA PROBES, as negative technical control. The PLA signal was
229 detected in a confocal microscope (Inverted Olympus MPhot) using a 60X
230 immersion oil objective and values determined considering the mean of three
231 images per condition in three independent experiments. Image analysis was
232 carried out using the Fiji ImageJ software, and the signal quantified as
233 described in Alcalá et al. (2017).

234 **Surface Plasmon Resonance:** Recombinant, human SRB1 (Sino Biological,
235 USA) was covalently immobilized onto carboximethylated-dextran sensor chips
236 (CM5 series S, General Electric Healthcare). Previously, the sensor chip
237 surface was activated using 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide
238 (EDC) and N-hydroxysuccinimide (NHS) reagents, according to the standard
239 procedure for amine coupling. For direct capture-coupling to NH₂ (in
240 immobilization buffer of 10 mM sodium acetate, pH 5.0) the SRB1 was diluted
241 to 30 µg/mL and injected at 5 µg/min flow-rate until reach a density of 10,000
242 resonance units (RU), using running buffer HBS-EP*. The remaining surface-
243 active groups were blocked with 1M ethanolamine hydrochloride, pH 8.5. A
244 dilution series of the DENV recombinant NS1 protein (Aalto Bio Reagents,
245 Dublin), at six concentrations of 23.4, 46.8, 93.7, 187.5, 375 y 750 nM, were
246 then injected at a flow rate of 30 µL/min for 200s of association with SR-B1,

247 followed by dissociation step adjusted to 600s. The surface was successively
248 regenerated between analyte injections with a single-brief (30s) injection of
249 glycine-HCl, pH 2.5. All analyses were carried out at 25° C using a Biacore
250 T200 (General Electric Healthcare). Surface plasmon resonance (SPR)
251 response data defined as sensorgrams, were zeroed at the beginning of each
252 individual injection and then double referenced. The responses were plotted
253 against the analyte concentration and fit to a 1:1 ($A+B = AB$) binding model
254 using Biacore T200 evaluation software, version 2.0 (General Electric
255 Healthcare).

256

257 Results

258 A scavenger-like receptor is expressed insect cells plasma membrane.

259 The expression of SRB1 in liver cells has been widely reported (Shen et al
260 2019). To evaluate the expression of SRB1 in C6/36 cells, non-permeabilized
261 C6/36 monolayers were immunolabeled with an antibody specific for human
262 SRB1 and analyzed by confocal microscopy, using Huh-7 and Vero cells as
263 controls. The results indicated that the SRB1 is expressed in the cell membrane
264 of Huh-7 and C6/36 cells, but not in the kidney derived Vero cells (Fig. 1A). To
265 further corroborate that C6/36 cells indeed express the SRB1 on the cell
266 surface, the location of the SRB1 on C6/36 was determined by TIRF
267 microscopy, using Huh-7 as positive controls. As shown in Figure 1B, a clear
268 signal for the SRB1 was obtained, indicating that C6/36 cells express a SRB1
269 molecule on the plasma membrane. Finally, it was inquired if a sequence for
270 this receptor was annotated in the genome of *Aedes albopictus*; a putative gene
271 for SRB1, encoding a protein with an estimated molecular weight of 58 Kd,
272 and a homology of 33.4 % with the human protein, was found in the NCBI
273 protein data base, under the code XP_019526049.2. These results taken
274 together indicate that C6/36 cells express an SRB1-like protein on the plasma
275 membrane.

276 DENV and ZIKV NS1 are efficiently internalized in hepatic and insect cells.

277 It is well established that DENV NS1 is internalized in human hepatic and
278 dendritic cells, rendering the cells more susceptible to DENV infection (Alcon-
279 LePoder et al, 2005; Alayli and Scholle, 2016); yet internalization of DENV NS1
280 in mosquito cells or of ZIKV NS1 in any cell type have not been reported. Thus,
281 using Huh-7 liver cells in parallel, we evaluated if DENV and ZIKV NS1 were
282 also internalized by C6/36 mosquito cells after 1 or 2 hours of incubation. As
283 shown in Figure 2, permeabilized cells were readily stained with anti-NS1
284 antibodies indicating that DENV and ZIKV NS1 were efficiently internalized by
285 both cell lines. However, different fluorescence patterns were observed for the
286 internalized DENV NS1 in liver and mosquito cells; while a punctuate pattern
287 was observed in liver cells, a diffuse cytoplasm distribution for NS1 was

288 observed in the mosquito cells. Likewise, different fluoresce patterns were
289 observed between internalized DENV and ZIKV NS1 in liver cells, with ZIKV
290 NS1 showing a fiber-like pattern. These observations, in agreement with
291 previous data (Puerta-Guardo, et al., 2019), suggest that there may be cell and
292 virus dependent differences in the way NS1 is internalized.

293 **DENV and ZIKV NS1 enhance the viral yield in insect cells:** Once the entry
294 of NS1 into C6/36 and Huh7 cells was assessed, next whether preincubation of
295 mosquito cells with ZIKV or DENV NS1 will render the cells more susceptible to
296 virus replication was evaluated. For this, C6/36 confluent monolayers were
297 incubated with 0, 3.5 or 7 $\mu\text{g}/\text{well}$ of NS1 for 1,5 h. Afterward, cells were
298 infected with DENV or ZIKV at a $\text{MOI} = 1$ and at 48 hpi the supernatants were
299 collected to determine the viral titer. A significant increase in the virus yield was
300 observed from DENV and ZIKV NS1 pre-incubated cells in comparison with
301 those not pre-incubated (Fig 3). The viral titers for DENV were 2.5×10^3 , 6.5×10^3
302 and 7×10^3 UFP/mL and for ZIKV, 4.5×10^4 , 1.5×10^5 and 1.8×10^5 UFP/mL in cells
303 pre-exposed to 0, 3.5 and 7 $\mu\text{g}/\text{mL}$ of NS1, respectively. The viral titer did not
304 experience a significant increase in cells pre-incubated with 3.5 $\mu\text{g}/\text{mL}$ or 7
305 $\mu\text{g}/\text{mL}$ of NS1, perhaps due to a saturation of the system. This result suggests
306 that exposure and internalization of NS1 by C6/36 cells before viral infection
307 favors viral particles production, in agreement with previous results reported for
308 liver and dendritic cells (Alcon-Le Poder et al., 2005; Alayli and Scholle, 2016).

309 **SRB1 participate in the entry of NS1 in hepatic and insect cells.** To
310 evaluate the participation of the SRB1 in the entry of NS1, monolayers of C6/36
311 or Huh7 cells were treated with 0, 1 and 10 $\mu\text{g}/\text{mL}$ of anti-SRB1 monoclonal
312 antibody to block the SRB1. After removal of the antibody, cells were incubated
313 with DENV NS1. Cells were fixed, permeabilized and immunolabeled to detect
314 internalized DENV NS1 as described above. Fluorescent signals were
315 quantified to compare the internalization of the NS1 in the presence or absence
316 of the antibody. A significant decrease of approximately 30%, in the intracellular
317 specific signal for NS1 was observed in Huh7 cells pre-incubated with the
318 specific monoclonal antibody compared to controls, no pre-incubated cells. No
319 significant difference in the signal was observed when the cells were pre-
320 incubated with 1 or 10 $\mu\text{g}/\text{mL}$ of the antibody (Fig. 4A and B). A significant
321 reduction in the internalization of DENV NS1 was also observed in C6/36 cell
322 treated with the anti-SRB1 antibody (Fig. 4C and D). The specific intracellular
323 fluorescent signal for DENV NS1 in C6/36 cells, showed a decrease of
324 approximately 38% and 63% when preincubated with 1 or 10 $\mu\text{g}/\text{mL}$ of the anti-
325 SRB1 antibody, respectively, in comparison with the control. The significant
326 decrease in the intracellular NS1 specific signal observed in the preincubated
327 cells suggests that blocking SRB1 with specific antibodies affects the entry of
328 NS1 in both cell lines.

HDL is the natural ligand of SRB1 in human liver cells. In order to evaluate if the natural ligand of the SRB1 will compete with NS1, monolayers of Huh-7 and C6/36 cells were pre-incubated with human HDL at 0, 75 or 150 µg/mL. After several washes to remove unbound HDL, the monolayers were incubated with 3,5 µg/well of DENV NS1 and for this competition studies, ZIKV NS1 was also included, at the same concentration. Huh-7 cells showed a reduction of approximately 28 and 35% in the amount of internalized DENV NS1. In addition, the incubation of Huh-7 cells with HDL also reduced the amount of internalized ZIKV NS1 in approximately 22 and 45%, depending on the HDL concentration (Fig. 5A). Interestingly, incubation of C6/36 cells with human HDL also caused significant reductions in the amount of internalized DENV and ZIKV NS1. With DENV NS1 reductions of approximately 64 and 76% were observed and with ZIKV NS1 reductions of approximately 81% and 86% were observed, depending on the HDL concentration (Fig. 5B). These results are in agreement with the results obtained with the anti-SRB1 antibodies and indicate that SRB1 act as the cell receptor for both DENV and ZIKV NS1 in vertebrate and mosquito cells. Of note, attempts to knock down the expression of the SRB1 in Huh-7 cells using siRNAs were unsuccessful since in our hands, transfection of the siRNAs specific for the SRB1, resulted highly toxic for these cells.

Overexpression of the SRB1 in Vero Cells. We observed previously that Vero cells do not express the SRB1 (Fig. 1). In order to further demonstrate the participation of the SRB1 in the DENV NS1 internalization process, a gain of function experiment, overexpressing the SRB1 in Vero cells was conducted. Confluent monolayers of Vero cells were lipo-transfected with a plasmid expressing the SRB1. The expression of the SRB1 were determined at 24 hpt by confocal microscopy. Despite the different conditions tested to express the SRB1, the maximum percentage of cells expressing the SRB1 without cell damage, was around 7%, observed at 24 hpt when combining 0,5 µg/well of the plasmid with 2µL of transfectant reagent (Fig. 6). Under these conditions, cells were incubated with DENV NS1, fixed, permeabilized and simultaneously immunolabeled for SRB1 and DENV NS1. As shown in Figure 6, internalization of DENV NS1 was observed only on those cells expressing the SRB1. These data indicate that the sole expression of the SRB1 is enough to render Vero cells capable of internalizing DENV NS1.

SRB1 interacts with DENV and ZIKV NS1 protein *in vivo* and *in vitro*. To determine if there is actual physical interaction between DENV and ZIKV NS1 and the SRB1, the interactions between them on C6/36 and Huh7 cells was analyzed *in situ* by PLA. The assay clearly revealed that the DENV and ZIKV NS1 interact with the SRB1 in both cell lines (Fig. 7A, C, E and G). Meanwhile, no signal was observed in the technical controls run in parallel (Fig. 7 B, D, F, H), showing the specificity of the red dots signal observed.

370 The second approximation to evaluate DENV NS1 and SRB1 physical
 371 interactions was SPR. As shown in the sensorgram in Figure 8, the SRB1
 372 developed a faster on-rate (0 to 200s) and slower off-rate (200s and on) with
 373 the interacting recombinant DENV NS1, at all the NS1 concentrations tested.
 374 The DENV NS1 showed a relative high affinity towards the SRB1, as indicated
 375 by the calculated average equilibrium dissociation constant (KD) values,
 376 ranging in the sub-nanomolar range (47.02 ± 0.01 nM versus 10mM or higher
 377 for non-specific binding interactions). Of note, the average equilibrium
 378 dissociation constant value observed for the recombinant NS1 and the SRB1 is
 379 in the same order of magnitude as those observed for the HDL and the SRB1
 380 (KD=18.71 nM). The SPR results corroborate the results obtained in the infected
 381 cells by PLA, and together indicate that the SRB1 is bound by the DENV, and
 382 ZIKV NS1 protein.

383 Discussion

384 NS1 is one of the most pleiotropic protein encoded by flaviviruses described so
 385 far. The ability of DENV NS1 to be secreted concomitantly with viral particles
 386 and reaching the extracellular milieu makes it a powerful “viral weapon” capable
 387 of participating in several pathogenesis processes, both intracellularly and
 388 extracellularly, thus favoring the installation and progression of the infection.
 389 DENV NS1 is internalized in cultured cells as well as *in vivo*, out of the context
 390 of the viral infection, to enhance viral production. Also, the endothelium
 391 disrupting capacity of flavivirus NS1 is dependent on cell internalization.
 392 However, little it is known about the mechanisms and host molecules involved
 393 in NS1 internalization. In this work, we assessed the participation of the SRB1
 394 as a receptor of the DENV and ZIKV NS1 in human hepatic and insect cells.
 395 First, we observed the internalization of DENV and ZIKV NS1 in hepatic cells as
 396 previously reported (Alcon-Le Poder et al., 2005, Puerta-Guardo et al., 2019).
 397 Additionally, we observed that NS1 internalization also occurs in insect cells.
 398 Moreover, as has previously been reported for human hepatic and dendritic
 399 cells, preincubation of insect cells with DENV or ZIKV NS1 renders the cells
 400 more susceptible to the respective viral infection. The mechanisms by which
 401 NS1 contribute to increase the viral yield it is not known. However, it has been
 402 proposed that the accumulation of DENV NS1 in the late endosomal
 403 compartment after the entry to hepatocytes potentializes subsequent dengue
 404 virus infection *in vitro* (Alcon-Lepoder et al., 2005). In addition, the entry of NS1
 405 to endothelial cells disrupt glycocalyx components which may favor virus
 406 binding and entry or potentially influencing virus dissemination and
 407 pathogenesis (Puerta-Guardo et al., 2016). In the mosquito cell, NS1 may also
 408 negatively regulate the innate cell response favoring viral replication (Liu et al.,
 409 2017). These mechanisms are not mutually excluding and may be all operating
 410 in the different cell types. In any case, additional experiments are needed to

411 determine what are the mechanisms leading to increase DENV and ZIKV
412 replication after NS1 cell intake.

413 The entry of a protein into a cell is a multifactorial process since the plasmatic
414 membrane is a plethora of different kind of molecules determined by the origin
415 of the cell, which in turn modulate the nature of proteins to be internalized
416 (Walrant et al., 2017). In hepatic cells, SRB1 is the main receptor of the HDL,
417 playing an important role in the homeostasis of lipoproteins circulating in serum
418 (Shen et al 2019). Derived from the fact that NS1 is internalized in liver cells
419 and is a lipoprotein in nature, in this work we present data indicating the
420 participation of the SRB1 in the internalization process of DENV and ZIKV NS1
421 in hepatic and insect cells. Our results indicate that a functional SRB1-like
422 receptor is encoded in the mosquito genome and expressed on the surface of
423 the cell plasma membrane. As mentioned, the main function of the SRB1 is to
424 participate in the influx and efflux of the HDL hauled molecules contributing to
425 lipid homeostasis. Mosquito cells are unable to synthesize cholesterol *de novo*
426 and SRB1-like receptor may participate in cholesterol uptake by these cells. On
427 the other hand, our results showing the absence of the SRB1 on kidney derived
428 Vero cells, agrees with previous results where the presence of NS1 was detected
429 in the liver but not in the kidneys of mice exposed intravenously to DENV NS1
430 (Alcon-Lepoder et al., 2005).

431 The entry of DENV NS1 both in hepatic and insect cells was hampered by the
432 preincubation with a specific monoclonal antibody against the SRB1. In both
433 cells, there was a significant reduction of the NS1 internalization when 1 or 10
434 µg/mL of the antibody was used. However, the antibody only partially abolishes
435 the NS1 binding and internalization, especially in hepatic cells, even at the
436 higher concentrations used. It has been reported that position C323 plays a
437 critical role in HDL binding and that the exoplasmic C384 plays a role in the
438 selective HDL-cholesteryl esters (CE) uptake and in SRB1-mediated selective
439 HDL-CE transport activity (Guo et al., 2011, Yu et al., 2011). The antibody used
440 in this work was developed against an epitope near the amino-terminal region of
441 the SRB1, away from positions C323 and C384. Thus, the antibody may only
442 partially cause a steric impediment for NS1 internalization. Also, a significant
443 but incomplete competition between NS1 and HDL, the natural ligand of the
444 SRB, was observed. These results indicate that the SRB1 is used as a cell
445 receptor by NS1. The participation of the SRB1 as cell binding molecule for NS1
446 was corroborated by the results obtained with transfected Vero cells where the
447 sole expression of SRB1 is enough to render the cell susceptible to NS1 entry.
448 Yet if HDL and NS1 bind to the same domains on the SRB1 molecule is
449 unknown. In addition, as is the case for HCV entrance, where the successful
450 binding in hepatic cells requires the SRB1, claudin-1, the human cluster
451 differentiation CD81 and occludin as a minimal set of receptors working

orchestrated as a multimolecular complex (Miao et al., 2017), SRB1 may not be the only receptor molecule for NS1.

To point a given molecule as a receptor for a ligand, evidence of direct interaction between the molecule and the ligand is required. In this work, direct physical interaction between the SRB1 and the DENV and ZIKV NS1 in Huh-7 and C6/36 cells was demonstrated by PLA. PLA is a relatively novel technique that uncover protein-protein interactions among proteins located not farther than 40 nm apart, and at variance with immunoprecipitation techniques, it does so maintaining the cell architecture (Sable et al., 2018). Moreover, PLA are run in parallel with several negative controls to assure the specificity of the reaction. Recently, the importance of NS1 present in the blood meal taken from the mammalian host to enhance the dissemination of ZIKV in the mosquito was reported (Liu et al., 2017). Thus, it is likely that the SRB1-like receptor expressed in mosquito cells is one of the molecules recognized by ZIKV NS1 upon blood ingestion. The PLA results for DENV NS1 and the SRB1 were corroborated *in vitro* by SPR. The results obtained by SPR clearly indicate physical interactions between the DENV NS1 and the SRB1 in a dose-dependent manner. Interestingly, the use of HDL in the SPR assays as a positive control showed that the affinity of the DENV NS1 and the human HDL for SRB1 does not differ significantly. This observation and the observation that HDL and DENV NS1 compete for the same receptor in Huh-7 cells, together with the high levels of circulating NS1 seen in patients during the acute phase of the disease, offers an explanation for the alterations in lipid homeostasis seen in dengue patients, as for example the decreased in HDL levels observed in patients with severe dengue (Li et al 2013).

In summary, in this work evidence is presented that the SRB1 in hepatic cells and an SRB1-like receptor in mosquito cells act as the cell binding protein for DENV and ZIKV NS1. Moreover, evidence is presented indicating that the C6/36 receptor is functional. It had been described that the selective uptake of cholesteryl esters by the cells through the SRB1, either *in vitro* and/or *in vivo*, do not show any specificity toward a single class of lipoproteins (Shen et al, 2018). Thus, we hypothesize that NS1 hijacks classic lipid routes for cell entry through the SRB1, but if the lipids carried out by NS1 are transferred to the cell is unknown. Once inside the cell, NS1 promotes viral replication and cause delocalization of tight junction proteins. Thus, future experiments should include the elucidation of the signaling pathways that are triggered after the SRB1 and NS1 interactions.

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Figure 1. Detection of the scavenger receptor B1 (SRB1) in liver, mosquito and kidney derived cell lines. A) Confluent cell monolayers were fixed, stained for SRB1 (red), the nuclei counterstained with DAPI (blue) and observed by confocal microscopy. B) The presence of the SRB1 on the cell plasma membrane was corroborated by Total Internal Reflection Fluorescence (TIRF) Microscopy, costaining cells for SRB1 (red) and wheat germ agglutinin (green) as cell membrane marker.

Figure 2. Internalization of DENV and ZIKV rNS1 in Huh-7 (A) and C6/36 cells (B). Cells were preincubated with recombinant NS1 and at the indicated times cells were washed, fixed, permeabilized and stained for intracellular NS1 (red), nuclei counterstained with DAPI and observed by confocal microscopy.

Figure 3. DENV (A) and ZIKV (B) titers after preincubation or not of C6/36 cells with recombinant NS1. Monolayers of C6/36 cell were preincubated for 1,5h with the indicated concentrations of either DENV or ZIKV NS1. After washing, cells were infected with the corresponding virus at a MOI=3 for 1h, infection allowed to proceed and the supernatants collected at 24 hpi, titrated by plaque assays in Vero cells. * $p=0.05$.

Figure 4. Inhibition of NS1 internalization by antibodies against the SRB1. Huh7 cells (A,B) and C6/36 (C,D) cells were incubated with anti-SRB1 antibodies at the indicated concentrations for 1h. After extensive washing, cells were incubated with DENV and ZINK NS1 (3,5 μ g/well) for 90 min. Finally, cells were fixed, permeabilized and labelled for NS1. Cells were analyzed by confocal microscopy (left panels). The levels of NS1 inside the cells and expressed as fluoresce arbitrary units (FAU) (right panels). Differences in FAU were compared for significance using an ANOVA test. * $p=0.05$. $n=3$.

Figure 5. Competition assays between human HDL and recombinant DENV and ZIKV NS1. A) Huh-7 (A) and C6/36 cell (B) cells were incubated with HDL at the indicated concentrations for 90 min; after extensive washing, cells were incubated with DENV and ZINK NS1 (3,5 μg/well) for 90 min. Finally, cells were fixed, permeabilized and labelled for NS1. Cells were analyzed by confocal microscopy (left panels). The levels of NS1 inside the cells and expressed as fluoresce arbitrary units (FAU) (right panels). Differences in FAU were compared for significance using an ANOVA test. * $p=0.05$. $n=3$.

Figure 5, cont.

Figure 6. Expression of the SRB1 receptor in Vero cells. Vero cells were transfected with a plasmid encoding the SRB1 and 24 hpt incubated with recombinant DENV NS1 for 90 min. Cells were fixed, permeabilized and co-immunolabeled with anti-NS1 (green) and anti-SRB1 (red) and analyzed by confocal microscopy. Nuclei were counterstained with DAPI.

Figure 7. Proximity ligation assays for dengue and zika virus NS1 and the SRB1 in human and mosquito cells.

Cells incubated with recombinant DENV NS1 (panels A, B, C and D) or ZIKV NS1 (panels E, F, G and H) at a concentration of 3,5 $\mu\text{g}/\text{well}$ for 45 min, then fixed and processed following the manufacturer's instructions. Positive interactions between NS1 and SRB1 are visualized as red dots. Cell nuclei were counterstained with DAPI. As negative controls, cells were preincubated with recombinant NS1, but primary anti-SRB1 antibody omitted (panels B, D, F and H). Experiments were carried out three times and typical results are shown.

Figure 8. Surface plasmon resonance sensorgrams obtained for the binding interaction of NS1 with the SRB1. Increasing concentrations (color lines) of recombinant NS1 were tested for interaction with SRB1 immobilized on a Biacore sensor chip. Binding curves were expressed in resonance units (RU) as a function of time (seconds). Solid black lines through the curve show model fit for the calculation of interaction affinities.