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Heparan sulfate facilitates Rift Valley fever virus entry into the cell

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RUNNING TITLE

Heparan sulfate-dependent entry of a *Phlebovirus*

KEYWORDS

bunyavirus, heparan sulfate, receptor, attachment factor, entry, *Phlebovirus*

16 ABSTRACT

17 **Rift Valley fever virus (RVFV), an emerging arthropod-borne pathogen, has a broad host and**
18 **cell tropism. Here we report that the glycosaminoglycan heparan sulfate, abundantly present**
19 **on the surface of most animal cells, is required for efficient entry of RVFV. Entry was**
20 **significantly reduced by preincubating the virus inoculum with highly-sulfated heparin, by**
21 **enzymatic removal of heparan sulfate from cells and in cells genetically deficient in heparan**
22 **sulfate synthesis.**

23

MAIN TEXT

Rift Valley fever virus (RVFV) belongs to the *Phlebovirus* genus of the *Bunyaviridae* family. Its negative-stranded tripartite RNA genome is encapsidated by nucleocapsid protein and is surrounded by a lipid-containing envelop which is derived from the trans-Golgi network (36). Two membrane-anchored viral glycoproteins, Gn and Gc, assemble into capsomers that cover the viral surface following a T=12 icosahedral symmetry (12, 19). The glycoproteins mediate host cell attachment of the virus and its subsequent entry into the cell (36). A 78-kDa glycoprotein of unknown function, which is an N-terminally extended version of Gn, has been reported as a third structural glycoprotein, present only in minute amounts in the viral envelop (21, 39).

RVFV is responsible for severe epidemics among ruminants in Africa and on the Arabian Peninsula, manifested by abortion storms and high mortality among young animals. The virus is transmitted by a wide variety of mosquito vectors. After introduction into the body by the bite of an infected mosquito, the virus can spread and infect different organs including the brain (32). Humans can also be infected of which a small percentage develops severe disease (31, 36). Apart from mosquitoes, ruminants and humans, a wide variety of animal hosts can be infected with RVFV including nonhuman primates, rodents and pets (11, 18). The virus also efficiently infects a large collection of different cell types in vitro (Fig. S1). The broad host, tissue and cell tropism of RVFV suggests the involvement of a common cell surface attachment factor to be utilized by RVFV to establish infection.

To initiate entry into the cell, viruses need to interact with a cellular receptor, which is sometimes preceded by binding to a primary attachment factor (30). The cell surface structures which facilitate entry of bunyaviruses remain largely unknown, although some receptors have been described. Beta3 integrins and nucleolin have been reported to be involved in attachment of *Hantavirus* and Crimean-Congo hemorrhagic fever virus (genus *Nairovirus*), respectively (14, 42). DC-SIGN, a C-type lectin primarily restricted to interstitial dendritic cells and certain tissue macrophages (33), has been identified as a receptor for some *Phleboviruses* including RVFV (29). The broad cell tropism of RVFV, however, suggests that other receptors are important for virus entry into cells that lack DC-SIGN expression.

All eukaryotic cells are covered by a dense and diverse array of carbohydrates. These sugars are essential for many different biological processes (40). It is not surprising that many viruses have

53 evolved to use these ubiquitous and accessible surface glycans as part of their strategy to infect cells
54 (26). Two types of glycans, sialylated glycans (SG) and glycosaminoglycans (GAGs), have been
55 particularly reported to play a role in virus entry. For example, influenza viruses specifically binds SGs,
56 while dengue virus (7) and adenovirus (34) interact with GAGs to facilitate entry. Merkel cell
57 polyomavirus has been reported to use both SGs and GAGs for entry (37).

58 We started to study the involvement of SGs and GAGs in RVFV entry by using a collection of Chinese
59 hamster ovary (CHO) cell mutants with specific genetic deficiencies in glycan synthesis (Table S1)
60 (22). Thus, CHO lec1 and 15B (16, 38) mutants are incapable of synthesizing complex N-linked
61 glycans, while the CHO lec2 mutant cells express sialic acid-free N- and O-linked glycans (9). The
62 CHO pgsA-745 cell mutant (10) is deficient in the synthesis of GAGs. To facilitate our studies, we
63 made use of the recently developed non-spreading RVFV (here referred to as $RVFV_{ns}$) (25). In
64 contrast to wild type virus, $RVFV_{ns}$ can be handled outside biosafety level-3 facilities, while the
65 presence of the eGFP gene in the viral genome enables infection to be easily monitored. The mutant
66 CHO cells, lec1 and 15B, and to a somewhat lesser extent the CHO lec2 cells were as efficiently
67 infected with $RVFV_{ns}$ as the parental wild type cells (pro5 and K1), suggesting that N- and O-linked
68 SGs play a minor role in virus infection. On the contrary, infection of CHO psgA-745 was dramatically
69 reduced, indicating that GAGs are important for $RVFV_{ns}$ infection (Fig. 1).

70 GAGs are linear polysaccharides that can be attached to proteins to form proteoglycans. There are
71 five classes of GAGs, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan
72 sulfate, and hyaluronic acid (28). Of these GAGs, HS has been identified as an attachment factor for a
73 number of viruses and is abundantly expressed on most cell types unlike other GAGs (28). We first
74 evaluated whether $RVFV_{ns}$ infection could be inhibited by including soluble heparin, a GAG analogue
75 of HS, as a competitor in the inoculum (23). As a control virus we used a non-spreading vesicular
76 stomatitis virus (here referred to as VSV_{ns}), a VSV-ΔG/GFP recombinant virus pseudotyped with its
77 authentic fusion glycoprotein G (5). Preincubation of $RVFV_{ns}$ with heparin reduced infection on CHO
78 K1 cells in a dose-dependent manner, whereas for VSV_{ns} no such effect was observed (Fig. 2A). To
79 confirm the involvement of HS in RVFV entry, CHO K1 cells were treated prior to infection for 1 hour at
80 37°C with different heparinases or chondroitinase to remove HS or CS/DS, respectively, from the cell
81 surface (Fig. 2B). Enzymatic treatment of CHO K1 cells with heparinase caused a marked increase of
82 infection with VSV_{ns} of more than 2-fold. In contrast, independent of the different heparinases used,

infection of heparinase-treated cells with $RVFV_{ns}$ was reduced to about 50%. No effect of chondroitinase treatment was observed. The reduced infectivity of $RVFV_{ns}$ of heparinase-pretreated cells was confirmed using six different cell lines, while the susceptibility of these cells to VSV_{ns} was not affected (Fig. S2).

To further characterize the interaction between RVFV and highly sulfated HS polysaccharides, we analysed $RVFV_{ns}$ infection of CHO K1 cells passaged in the presence of 50mM sodium chlorate ($NaClO_3$). $NaClO_3$ is known to inhibit the addition of *O*-sulfate groups to GAGs (1, 35). Importantly, we did not observe any apparent changes in growth rate or cell morphology of CHO K1 or A549 cells cultured for 7 days in the presence of up to 70mM $NaClO_3$ (data not shown). Infection by $RVFV_{ns}$ of CHO K1 or A549 cells maintained in the presence of $NaClO_3$ was dramatically reduced (Fig. 3A and B), in contrast to infection by VSV_{ns}, suggesting that *O*-sulfation of HS is necessary for efficient $RVFV_{ns}$ infection of both cell lines.

Next we tested the susceptibility of CHO pgsD-677 cells (CHO HS[-]), which are deficient in HS synthesis (27), to $RVFV_{ns}$ and VSV_{ns} infection. Compared to its parental CHO K1 cells, infection of CHO HS(-) cells with $RVFV_{ns}$ was greatly reduced (>97%), whereas VSV_{ns} infection of these cells was enhanced (Fig. 4). To confirm HS dependency of RVFV, an autonomously replicating virus was included in this experiment. This virus expresses the eGFP reporter from its genome, similar to $RVFV_{ns}$, and was rescued as previously described (25). Also this virus displayed a significantly reduced infectivity on CHO HS(-) cells. Altogether the observations strongly support an important role of HS for RVFV infection.

Many viruses have been reported to utilize HS for host cell attachment (reviewed in (28)). Interactions of viruses with heparan sulfate are often based on electrostatic contacts between the negatively charged sulfate groups on HS and clusters of basic residues occurring in viral surface proteins. These clusters often comprise a BBXB or a BBBXXB motif (B, basic amino acid; X, any amino acid) (3). When analysing the complete M segment-encoded polypeptide sequence of the RVFV used in this study (strain 35/74, GenBank accession number JF784387.1), we identified two overlapping BBBXXB HS binding motifs (116-**R**CE***RRR***DA*K*-124) in the pre-Gn region of the 78-kDa protein while no HS binding motifs were identified in the Gn or Gc protein sequence. The 78-kDa protein is considered to be a minor structural glycoprotein (39) and is apparently dispensable: RVFV recombinants lacking the

pre-Gn region display wild-type growth kinetics in cell culture calling into question whether the basic amino acid motifs in the protein indeed contribute to HS binding (15, 41). Alternatively, other linear or non-linear arrangements of basic residues in Gn and/or Gc may create an HS binding motif in the tertiary structures of these glycoproteins (13, 17). Clearly, the identification of the HS binding site on the viral surface requires further study.

HS dependency has for some viruses been shown to be acquired after repetitive virus passage in cell culture through the acquisition of single or multiple amino acid substitutions in the surface glycoproteins, creating a positively charged HS-binding motif (6, 8, 20, 24). The RVFV strain 35/74 has been isolated from the liver of a sheep that died during an RVFV outbreak in the Free State province of South Africa in 1974. The virus was amplified in suckling mouse brain and passaged three times in BHK-21 cells (25). To study the possible acquisition of a HS-binding motif during these procedures the M segment encoded polypeptide sequence was aligned with those of four RVFV isolates that had been directly sequenced from serum or organ material of infected animals (2, 4). This analysis did not reveal the presence of additional basic amino acids in the 35/74 sequence (Table S2), indicating that the requirement for HS for efficient entry of the RVFV used in this study is not likely the result of cell culture adaptation.

Although infection of RVFV in the GAG- and HS-deficient CHO cells was dramatically reduced we observed residual infection of both cell lines. It remains to be determined whether this infection in the absence of HS is explained by the binding of RVFV to another, unidentified attachment factor or receptor present on these cells.

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FIGURE LEGENDS

FIG 1. $RVFV_{ns}$ infection is drastically reduced in the absence of GAGs. The CHO 15B and CHO 745 mutant cells derived from the CHO K1 cell line and the CHO lec1 and CHO lec2 mutant cells derived from the CHO Pro5 cell line were cultured in Ham's F-12K medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). Subconfluent monolayers were infected with $RVFV_{ns}$ at different moi's (0.12 and 0.6). Twenty hours post infection (hpi) the cells were washed once with PBS and prepared for fluorescence microscopy (A) or FACS analysis (B). (A) Cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature and representative pictures were taken using the EVOS fl microscope (AMG, magnification 4x; data shown refer to infections at moi of 0.6). Nuclei were counterstained with DAPI. MOCK represents mock-infected cells. (B) Cells were trypsinized and fixed with 3.7% formaldehyde in PBS for 20 minutes at room temperature and $RVFV_{ns}$ infected (GFP-positive) cells were quantified by FACS (FACS Calibur). Graphical data shown are normalized to the infectivity of CHO K1 or CHO Pro5 cells and are representative of two independent experiments performed in triplicate. Significant differences between conditions are indicated (ANOVA-Bonferroni) with *** corresponding to $p<0.001$. Error bars represent SD.

FIG 2. $RVFV_{ns}$ infection is decreased in the presence of heparin and after enzymatic removal of heparan sulfate from the cell surface. (A) $RVFV_{ns}$ and VSV_{ns} were incubated with different concentrations of soluble heparin (MPBio) for 10 minutes at room temperature in culture medium, prior to infection of CHO K1 cells. At 8 (VSV_{ns}) or 20 ($RVFV_{ns}$) hpi, infection was quantified by FACS as described for Fig. 1. The data shown correspond to a representative set of two independent experiments performed in triplicate. (B) GAGs were enzymatically removed from the cell surface of CHO K1 cells. Chondroitinase ABC (specific for chondroitin and dermatan sulphate), heparinase I (specific for heparin and highly sulfated domains), heparinase II (specific for heparin and heparan sulfate) and heparinase III (specific for heparan sulfate), all purchased at Sigma, were dissolved in resuspension buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 4 mM $CaCl_2$ and 0.01% BSA). Dilutions were prepared in digestion buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 4 mM $CaCl_2$ and 0.1% BSA). CHO K1 cells were treated for 1 hour at 37°C with heparinase I, II or III, a combination of them, or with chondroitinase at the indicated concentrations. The cells were washed twice with culture

medium, and then incubated with $RVFV_{ns}$ or VSV_{ns} for 30 minutes at 37°C. The cells were washed twice with culture medium and further incubated in culture medium at 37°C for 8 (VSV_{ns}) or 20 ($RVFV_{ns}$) hours after which infection was quantified by FACS as described for Fig. 1. Data were obtained from two independent experiments performed in duplicate. Significant differences between conditions are indicated (ANOVA-Bonferroni) with *** corresponding to $p<0.001$. Error bars represent SD.

FIG 3. $RVFV_{ns}$ infection strongly depends on sulfation of heparan sulfate. (A) CHO K1 cells were passaged twice in culture medium containing 50 mM $NaClO_3$ (Sigma) and subsequently cultured in the presence of 50mM sodium chlorate, or in chlorate-free culture medium for 30 or 8 hours prior to infection, to reverse the chlorate effect. Twenty hours post infection, cells were analysed by fluorescence microscopy or FACS as described for Fig. 1. Graphical data shown are normalized and are representative of two individual experiments performed in triplet. (B) A549 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS). CHO K1 or A549 cells were passaged twice in culture medium containing 50 mM $NaClO_3$ (Sigma) and subsequently cultured in the presence of 50mM sodium chlorate ($NaClO_3$ (+)), or in chlorate-free culture medium ($NaClO_3$ (-)) for 8 hours prior to inoculation with $RVFV_{ns}$ or VSV_{ns} at the indicated moi. Eight (VSV_{ns}) or twenty ($RVFV_{ns}$) hours post infection, cells were analysed by fluorescence microscopy or FACS as described for Fig. 1. Significant differences between conditions are indicated (ANOVA-Bonferroni, *** = $p<0.001$). Error bars represent SD.

FIG 4. Entry of $RVFV_{GFP}$ in GAG-deficient CHO cells is inefficient due to the lack of heparan sulfate. Mutant CHO cells pgsD-677 (HS[-], able to express all GAGs except for heparan sulfate) and pgsA-745 (CHO GAG[-], deficient in expression of all GAGs), and the parental CHO K1 cells were inoculated with $RVFV_{ns}$, VSV_{ns} or $RVFV_{GFP}$. At 8 (VSV_{ns}), 10 ($RVFV_{GFP}$) or 20 ($RVFV_{ns}$) hpi, cells were analysed by fluorescence microscopy and quantified for GFP expressing RVFV infected cells. Graphical data shown are normalized to the infectivity of CHO K1. Significant differences between conditions are indicated (ANOVA-Bonferroni) with *** corresponding to $p<0.001$. Error bars represent SD.

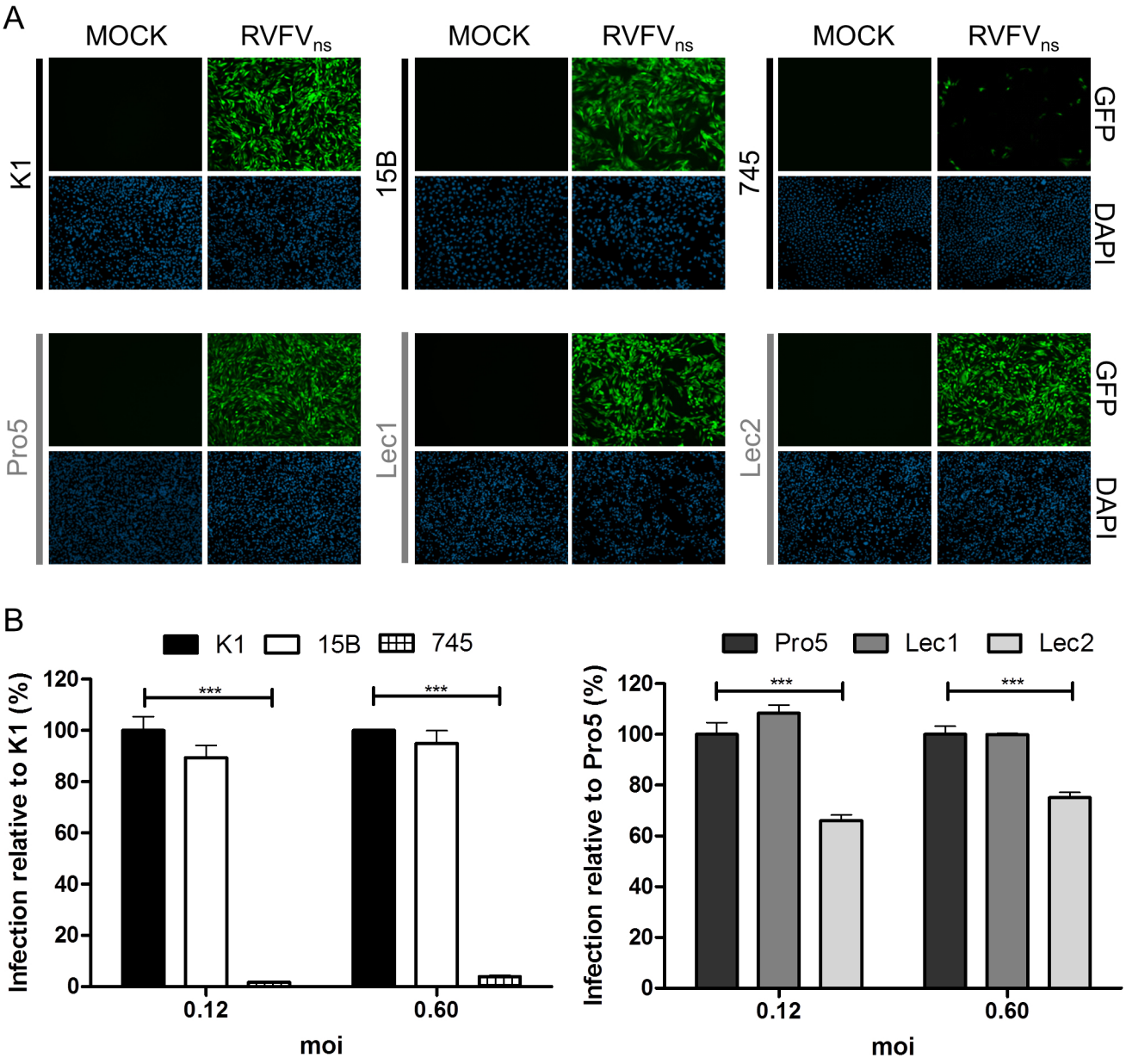

Fig 2.

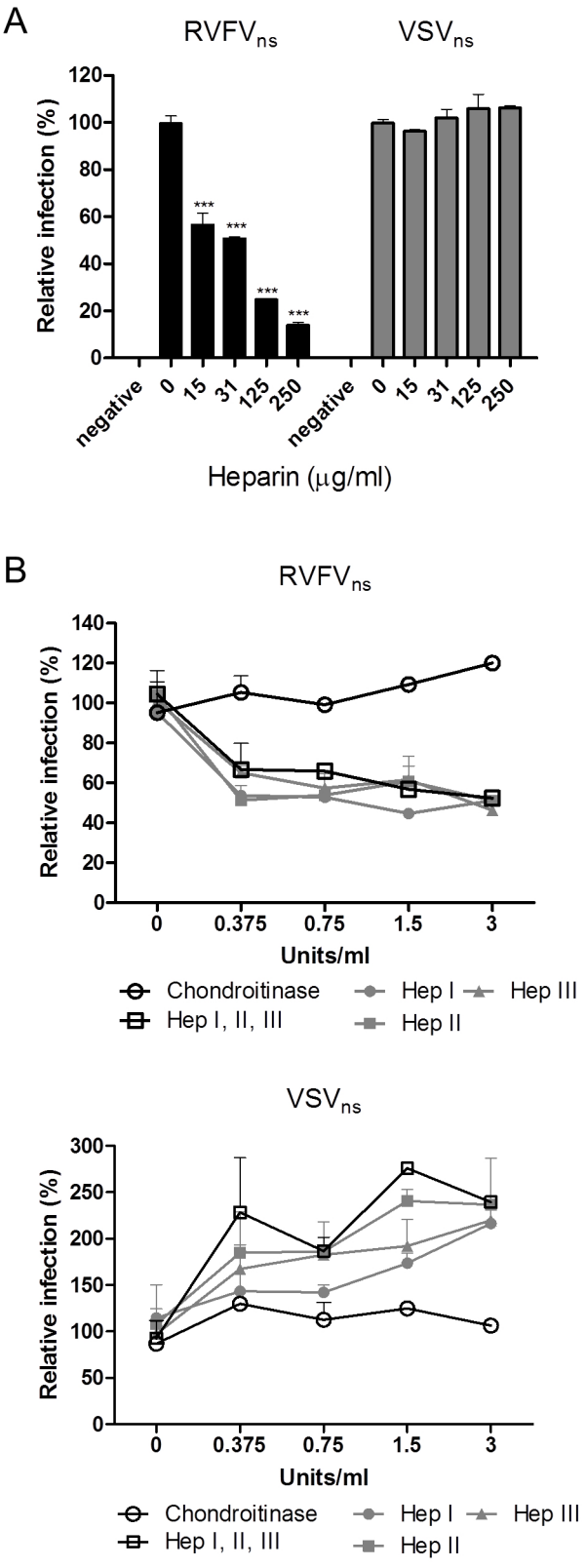

Fig. 3

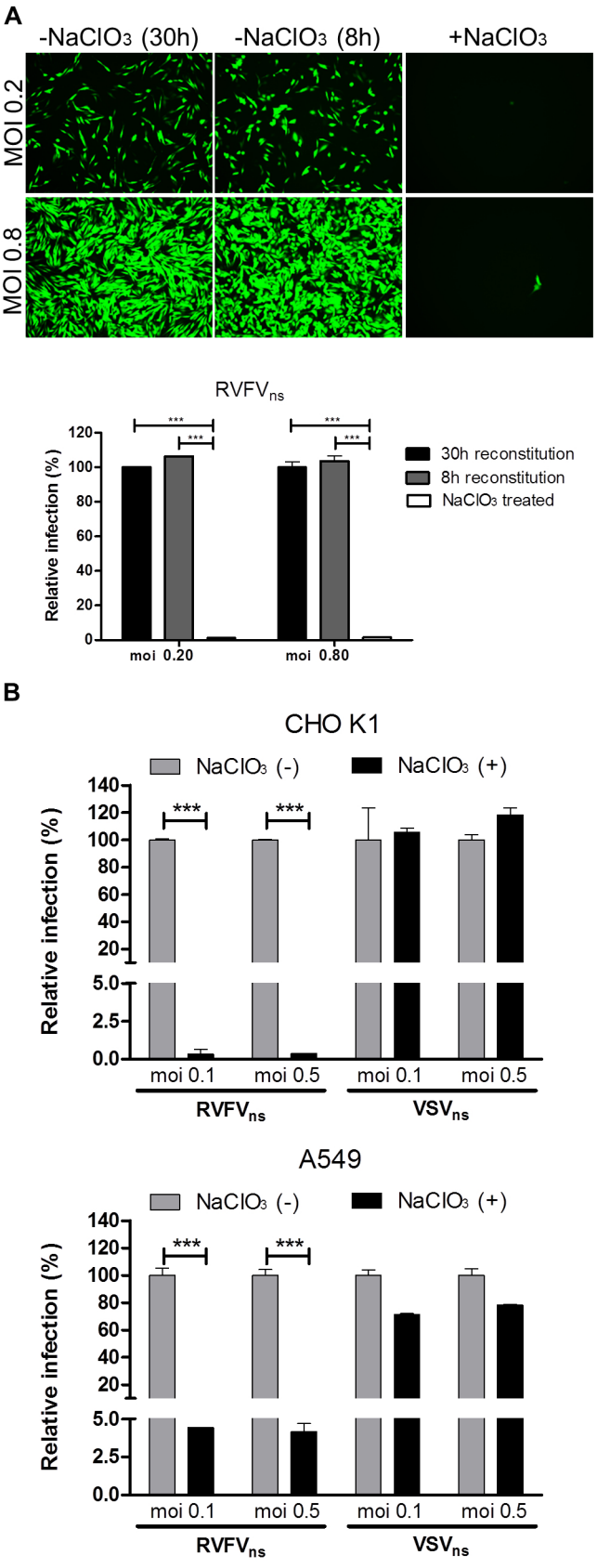

Fig 4.