



HOKKAIDO UNIVERSITY

Title	Characterization of the Envelope Glycoprotein of a Novel Filovirus, Lloviu Virus
Author(s)	Maruyama, Junki; Miyamoto, Hiroko; Kajihara, Masahiro et al.
Citation	Journal of virology, 88(1), 99-109 https://doi.org/10.1128/JVI.02265-13
Issue Date	2014-01
Doc URL	https://hdl.handle.net/2115/56456
Type	journal article
File Information	Maruyama et al.pdf



Characterization of the envelope glycoprotein of a novel filovirus, Lloviu virus

Junki Maruyama¹, Hiroko Miyamoto¹, Masahiro Kajihara¹, Hirohito Ogawa^{2,3}, Ken Maeda⁴, Yoshihiro Sakoda⁵, Reiko Yoshida¹, and Ayato Takada^{1,6*}

¹ Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

² Hokudai Center for Zoonosis Control in Zambia, School of Veterinary Medicine, The University of Zambia, Lusaka, Zambia

³ Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

⁴ Laboratory of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan

⁵ Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

⁶ School of Veterinary Medicine, the University of Zambia, Lusaka, Zambia

*Corresponding author; Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan

Tel.: +81-11-706-9502; Fax: +81-11-706-7310

E-mail: atakada@czc.hokudai.ac.jp

Key words: Filovirus, Lloviu virus, Ebola virus, Glycoprotein, virus entry

Running head: Characterization of a novel filovirus

Abstract: 228 words

27 **Abstract**

28 Lloviu virus (LLOV), a novel filovirus detected in bats, is phylogenetically distinct
29 from viruses in the genus *Ebolavirus* and *Marburgvirus* in the family *Filoviridae*. While
30 filoviruses are known to cause severe hemorrhagic fever in humans and/or nonhuman
31 primates, LLOV is biologically uncharacterized since infectious LLOV has never been
32 isolated. To examine the properties of LLOV, we characterized its envelope
33 glycoprotein (GP), which likely plays a key role in viral tropism and pathogenicity. We
34 first found that LLOV GP principally shares the primary structure with the other
35 filovirus GPs. Similarly to the other filoviruses, virus-like particles (VLPs) produced by
36 transient expression of LLOV GP, matrix protein, and nucleoprotein in 293T cells had
37 densely arrayed GP spikes on a filamentous particle. Mouse antiserum to LLOV VLP
38 was little cross-reactive to viruses of the other genera, indicating that LLOV is a
39 serologically distinct from the other known filoviruses. For functional study of LLOV
40 GP, we utilized a vesicular stomatitis virus (VSV) pseudotype system and found that
41 LLOV GP requires low endosomal pH and cathepsin L, and that human C-type lectins
42 act as attachment factors for LLOV entry into cells. Interestingly, LLOV
43 GP-pseudotyped VSV infected particular bat cell lines more efficiently than viruses
44 bearing other filovirus GPs. These results suggest that LLOV GP mediates cellular entry
45 in a manner similar to the other filoviruses while showing preferential tropism for some
46 bat cells.

INTRODUCTION

Filoviruses are nonsegmented, negative-stranded RNA viruses grouped into two genera, *Marburgvirus* and *Ebolavirus*. These filoviruses are known to cause severe hemorrhagic fever in human and/or nonhuman primates with case mortality rates of up to 90% (1). There is one known species of *Marburgvirus*, *Marburg marburgvirus*, consisting of two viruses, Marburg virus (MARV) and Ravn virus. On the other hand, five distinct species are known in the genus *Ebolavirus*; *Zaire ebolavirus*, *Sudan ebolavirus*, *Tai forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Tai forest virus (TAFV), Bundibugyo virus (BDBV), and Reston virus (RESTV), respectively. Among ebolaviruses, a difference in pathogenicity was suggested. EBOV is thought to be the most pathogenic, killing up to approximately 90% of patients, whereas RESTV has never caused lethal infection in humans (2) and is less pathogenic in experimentally infected nonhuman primates than EBOV (3).

Recently, a filovirus-like RNA genome was detected in the lungs, livers, rectal swabs, and/or spleens of bat (*Miniopterus schreibersii*) carcasses found in Cueva del Lloviu, Asturias, Spain. This novel filovirus was designated Lloviu virus (LLOV), whose name was derived from the cave in which it was first found (4). LLOV is phylogenetically distinct from other filoviruses and thus proposed to belong to the new genus *Cuevavirus*, species *Lloviu cuevavirus*, in the family *Filoviridae* (4). However, the biological properties of this novel virus are uncharacterized since infectious LLOV has not been isolated yet.

Filovirus particles consist of at least seven structural proteins, including the nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and

polymerase (L) genes (5). The envelope GP is responsible for both receptor binding and fusion of the virus envelope with the host cell membrane (6, 7). GP undergoes proteolytic cleavage by host proteases such as furin, resulting in the two subunits, GP₁ and GP₂, which are linked by a disulfide bond (7, 8). GP is highly glycosylated with large amounts of N- and O-linked glycans, most of which are located in its middle one-third, designated the mucin-like region (MLR), which plays an important role in attachment to the preferred target cells (9, 10). Although MLR is found in all known filovirus GPs, its highly variable amino acid sequences and sugar chain structures suggest different GP properties among filovirus species. Membrane-anchored cellular C-type lectins have been found to facilitate filovirus infection *in vitro* through binding to glycans on the MLR (11-13). It was also shown that MLR contains epitopes for antibody-dependent enhancement (ADE) of filovirus infection *in vitro* (14, 15).

To provide information for estimation of the infectivity and potential pathogenicity of LLOV, this study focused on GP, which likely plays major role in the replication cycle and the pathogenicity of filoviruses (10, 16). In this study, we investigated the morphology of virus-like particles consisting of LLOV GP, VP40, and NP, compared the antigenicity of GP among filoviruses, and analyzed the ability of GP to mediate virus entry into cells. Here we show that LLOV GP has the potential to mediate viral entry into cells of various animal species, including primates, in a manner similar to the other filoviruses while showing preferential tropism for some particular bat cells.

MATERIALS AND METHODS

Cells. Human embryonic kidney (HEK) 293, HEK293T, and African green monkey kidney Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and penicillin-streptomycin. Bat cell lines ZFB11-97 and SuBK12-08 were established by transfecting an expression plasmid encoding the Simian virus 40 large T antigen (pCXN2-Flag-SV40LT kindly provided by Drs. H. Sawa and Y. Orba, Hokkaido University Research Center for Zoonosis Control) into primary kidney cells of bats captured in Zambia. The transfected cells were selected by culturing in the presence of G418 (200 µg/ml). ZFB11-97, SuBK12-08, and Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) with 10% FCS, L-glutamine, and penicillin-streptomycin. SK-L cells (17) were cultured in MEM with 10% FCS, L-glutamine, and penicillin-streptomycin, and 0.3% tryptose phosphate broth (GIBCO). Bat cell lines BKT1, FBKT1, YubFKT1, IndFSPT1, and DemKT1, were established as described previously (18). Bat species were identified by morphology, habitat, and BLAST searches using the sequences of their cytochrome b genes (nucleotide positions 1-400). BKT1, FBKT1, YubFKT1, IndFSPT1, DemKT1, human chronic myelogenous leukemia (K562), and K562 clones expressing human macrophage galactose-type C-type lectin (hMGL) or dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (19, 20) were grown in RPMI-1640 medium with 10% FSC, L-glutamine and penicillin-streptomycin.

Construction of plasmids expressing GP, NP, and VP40. Coding regions of the GP, NP, and VP40 genes of LLOV were synthesized in pBS II SK vector (FASMAC) based on the nucleotide sequence of LLOV (GenBank accession number: JF828358). The NP

and VP40 genes were synthesized according to the coding regions reported in the database. Since the ebolavirus envelope GP is expressed through transcriptional editing (21, 22), the coding region of the GP gene was synthesized with an additional adenosine at the putative editing site. After digestion by restriction enzymes, each gene was cloned into mammalian expression vector pCAGGS (23). The expression plasmids for EBOV (strain Mayinga), SUDV (strain Boniface), TAFV (strain Cote d'Ivoire), BDBV (strain Bundibugyo), RESTV (strain Pennsylvania) and MARV (strains Angola and Musoke) were constructed as described previously (24).

Purification of virus-like particles (VLPs). HEK293T cells were transfected with plasmids encoding GP, VP40, and NP of LLOV, EBOV, SUDV, TAFV, BDBV, RESTV or MARV (strain Angola) using TransIT LT-1 reagent (Mirus) according to the manufacturer's instructions. Forty-eight hours later, VLPs were purified from culture supernatants by ultracentrifugation at 28,000 g at 4°C for 1.5 hours with a 25% sucrose cushion. VLP pellets were resuspended in phosphate-buffered saline (PBS).

SDS-PAGE and western blotting. HEK293T cells were transfected with plasmids encoding filovirus GPs and lysed 48 hours after transfection with a lysis buffer (10 mM Tris·HCl [pH 7.8], 0.15 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40 and protease inhibitor mixture)(Roche). Cell lysates were mixed with SDS-PAGE sample buffer with or without 5% 2-mercaptoethanol. After electrophoresis on 5–20% SuperSep (Wako), separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with 1:2000-diluted mouse antisera to filovirus VLPs (see below), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (H+L)

(Jackson ImmunoResearch). The bound antibodies were visualized with Immobilon Western (Millipore).

Mouse antisera and enzyme-linked immunosorbent assay (ELISA). Five-week-old female BALB/c mice were immunized twice intraperitoneally with purified VLPs (100 µg/mouse) at 3-week intervals. Antisera were collected 7 days after the second immunization. Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Hokkaido University Animal Care and Use Committee. The GP-based ELISA was performed as described previously (25). Serum samples were serially diluted with PBS containing 0.05% Tween 20, 0.5% bovine serum albumin, and 2% FCS. Bound antibodies were visualized by adding peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and 3,3',5,5'-tetramethylbenzidine (Sigma). The reaction was stopped by adding 1 N phosphate acid to the mixture, and the optical density (OD) at 450 nm was measured.

Electron microscopy. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out as described previously (26, 27). Purified VLPs fixed with 0.25% glutaraldehyde were adsorbed to a collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH=5.8). For immuno-TEM, we used an anti-LLOV GP monoclonal antibody (LGP14-2) produced in this study as described previously (15), and an immunogold-conjugated goat anti-mouse IgG (H + L) polyclonal antibody (BB International). Samples were examined with an H-7650 electron microscope (Hitachi) at 80kV. For SEM, cells transfected with

plasmids expressing LLOV GP, VP40, and NP were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in the same buffer. The fixed samples were dehydrated with a series of ethanol gradients, substituted with t-butanol, and dried in an ES-2030 freeze dryer (Hitachi). Dried specimens were coated with platinum by using Mild sputter E-1046 (Hitachi). The samples were observed with an S-4700 electron microscope (Hitachi) at 15 kV.

Vesicular stomatitis virus (VSV) pseudotyped with filovirus GPs. Using VSV containing the green fluorescent protein (GFP) gene instead of the receptor-binding VSV G protein gene (VSVΔG*-G)(6), pseudotyped viruses with GPs of EBOV, RESTV, MARV strains Angola and Musoke, and LLOV (VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Angola, VSVΔG*-Musoke, and VSVΔG*-Lloviu, respectively), were generated and the infectious units (IUs) of stock viruses were determined in Vero E6 cells, according to a previous study (6). The genome copy number of each pseudotyped VSV preparation was quantified by real-time RT-PCR. Real-time RT-PCR was performed using One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa Bio) and a CFX96 Real Time System (BIO RAD) with primers (GFP498-F: CAAGATCCGCCACAACATCG and GFP-617R: GACTGGGTGCTCAGGTAGTG) to detect the GFP gene in the VSV genome.

Virus titration. To determine infectivities of VSVs pseudotyped with filovirus GPs, appropriately diluted virus stocks were pretreated with an anti-VSV G monoclonal antibody, VSV-G(N)1-9, to abolish the background infectivity of parental VSVΔG*-G (15). K562 clones expressing hMGL and DC-SIGN grown on 96-well plates were

infected with VSV pseudotyped with filovirus GPs (50-150 IU determined in K562 cells), and infectivities were determined by counting the number of GFP-positive cells using flow cytometry as described previously (19, 20). For the assays of antibody-dependent enhancement (ADE) of infection, 10-fold serially diluted mouse antisera were mixed with equal volumes of pseudotyped VSVs (50-150 IU determined in K562 cells). After 1-hour incubation at room temperature, the mixture was inoculated into K562 cells grown on 96-well plates. At 20 hours post-inoculation, GFP-positive cells were counted with an IN Cell Analyzer 2000 (GE Healthcare). To determine the infectivities in adherent cells of different animal origins, cell monolayers grown on 96-well plates were infected with VSVs pseudotyped with filovirus GPs. Twenty hours later, the virus infectivity in each cell line was determined by counting the number of GFP-expressing cells under a fluorescent microscope, and IUs per 10^6 genome copies were calculated.

Inhibitor treatments. Vero E6 cells were pretreated with ammonium chloride (Wako), monensin (Sigma), cathepsin B, or L inhibitors (CA-074Me and FY-dmk, respectively, Calbiochem) for 30 min at 37°C. Treated cells were then infected with VSVΔG*-Zaire, VSVΔG*-Angola, VSVΔG*-Lloviu, and VSVΔG*-G appropriately diluted to yield 200–2000 IUs/ 10^6 cells. At 20 hours post-inoculation, GFP-positive cells were counted with the IN Cell Analyzer 2000 (GE Healthcare).

RESULTS

Characterization of the primary structure of LLOV GP. Though the envelope GPs of ebolaviruses are expressed through transcriptional editing (21, 22), similar characteristics were not shown for LLOV GP in a previous study, and the reported LLOV GP gene did not have an open reading frame of the single transmembrane GP (4). Thus, we first analyzed the nucleotide and deduced amino acid sequences of LLOV GP, and found that the nucleotide sequence of LLOV GP had 7 adenosines at positions 910-916. Based on sequence comparison with the EBOV genome, which has the editing site at positions 880-816, we assumed that this stretch of 7 adenosines could be the editing site of the LLOV GP gene. Accordingly, an open reading frame of the full length transmembrane GP gene was produced by adding an adenosine at this putative editing site (Fig.1A). We found that LLOV envelope GP had a potential cleavage site (i.e., RRRR recognized by host ubiquitous proteases such as furin) and MLR, similarly to the other filoviruses. The predicted MLR of LLOV GP differed from that of EBOV GP in length and location (i.e., LLOV MLR was located over the cleavage site and was a little shorter than EBOV MLR) (Fig. 1A and B). We confirmed the approximate molecular size of LLOV GP (GP_{1,2}, approximately 120-130 kD) and its cleavage product, the GP₁ subunit (approximately 100 kD), by western blotting (Fig. 1C). These results suggested that LLOV GP principally might share biological characteristics with the other filovirus GPs.

Morphology of VLPs consisting of LLOV GP, VP40, and NP. Filoviruses are characterized by their filamentous forms. However, LLOV particles have never been verified morphologically. To determine the possible shape of LLOV particles, we

investigated the morphology of VLPs consisting of LLOV GP, VP40, and NP. TEM analyses revealed that LLOV VLPs were filamentous (Fig. 2A and E), like EBOV (Fig. 2C and G) and MARV VLPs (Fig. 2D and H). Numerous spikes were observed on the VLP surface and immuno-TEM with an anti-LLOV GP monoclonal antibody confirmed the presence of LLOV GPs on the surface (Fig. 2B and F). Similarly to the other filoviruses (28), VLPs with a uniform diameter of approximately 70 nm and varied lengths were observed. This was consistent with a previous study showing that the diameters of VLPs were narrower than reported for actual EBOV particles (80-nm diameter) (1, 29). By SEM, numerous filamentous structures were observed on the surfaces of cells transfected with plasmids expressing LLOV GP, VP40, and NP (Fig. 3), which has a similarity to EBOV budding (27). These results suggested that LLOV shared morphological characteristics with the other known filoviruses.

Antigenic comparison among filovirus GPs. While LLOV is shown to be phylogenically distinct from the other filovirus species, serological information is lacking. The amino acid sequence of LLOV GP has 35% and 28% similarity with EBOV and MARV, respectively. To compare the antigenic relationships among filovirus GPs, we produced antisera to each GP by immunizing mice with VLPs, and performed GP-based ELISA (Fig. 4). We found that anti-LLOV GP sera showed exclusive reactivity to the LLOV GP antigen (Fig. 4A). Similarly, anti-MARV GP sera only reacted with the MARV GP antigen (Fig. 4G). Antisera to EBOV, SUDV, TAFV, BDBV, and RESTV GPs showed slight cross-reactivity with ebolavirus antigens at the lowest dilution of the sera but not with the LLOV and MARV GP antigens (Fig. 4B to

F). These results indicated that LLOV was distinct not only phylogenically but also serologically from the other known filoviruses.

Functional study of LLOV GP with chemical inhibitors. For functional study of LLOV GP, we produced VSV pseudotyped with LLOV GP, which could infect Vero E6 cells, and thus confirmed that the full length GP produced with 8 adenosines at the putative editing sites was fully functional as a single transmembrane GP. It has been shown that endosomal acidification and proteolytic processing with the cellular cysteine proteases cathepsin L and/or B are required for filovirus GP-mediated entry *in vitro* (6, 7, 30). To investigate the requirement of these factors for the LLOV GP function, we examined the infectivities of pseudotyped VSV in Vero E6 cells pretreated with chemical inhibitors (Fig. 5). Pretreatment of cells with ammonium chloride and monensin markedly reduced the infectivity of VSVΔG*-Lloviu, as was the case with VSVΔG*-Zaire, VSVΔG*-Angola, and VSVΔG*-G, suggesting that LLOV GP requires a low pH for cellular entry. We found that pretreatments with a cathepsin L inhibitor at concentrations of 2.5 and 10 μM significantly reduced the infectivities of VSVΔG*-Lloviu, -Zaire, and -Angola. However, treatments at 0.625 μM did not show any inhibitory effects on the VSVΔG*-Lloviu infectivity, and rather enhanced the infectivity. Interestingly, when cells were treated with a cathepsin B inhibitor, the infectivity of VSVΔG*-Zaire was reduced significantly in a dose-dependent manner, whereas much less inhibition were observed in the VSVΔG*-Lloviu, -Zaire, and -Angola infectivities. These cathepsin inhibitors did not affect the infectivity of VSVΔG*-G.

Human C-type lectin-mediated entry of pseudotyped VSVs. C-type lectins expressed on the host cell surface are thought to serve as an attachment factor for filovirus GP, and C-type lectin-mediated entry is believed to be one of the important factors responsible for filovirus tropism and pathogenicity (11, 19, 20). Thus, we investigated the potential of LLOV GP to use the human C-type lectins hMGL and DC-SIGN, both of which are known to enhance filovirus infectivity (Fig. 6). VSVΔG*-Lloviu infected DC-SIGN-expressing cells more efficiently than hMGL-expressing cells, which was similar to VSVΔG*-Zaire and VSVΔG*-Reston. VSVΔG*-Lloviu infected hMGL-expressing cells more efficiently than VSVΔG*-Musoke ($P < 0.05$), but less than VSVΔG*-Angola ($P < 0.05$), but there were no significant differences among VSVΔG*-Lloviu, VSVΔG*-Zaire, and VSVΔG*-Reston. On the other hand, VSVΔG*-Lloviu infected DC-SIGN-expressing cells more efficiently than VSVΔG*-Angola ($P < 0.05$) and VSVΔG*-Musoke ($P < 0.01$), and less efficiently than VSVΔG*-Zaire ($P < 0.05$), but there was no significant difference between VSVΔG*-Lloviu and VSVΔG*-Reston.

Difference in ADE activity between anti-EBOV and anti-LLOVGP antisera.

Antibody-dependent enhancement of infection is also a known *in vitro* phenomenon observed for comparatively highly lethal filoviruses (e.g., Zaire and Angola) (15, 31, 32). To investigate the potential of LLOV GP to induce ADE antibodies, K562 cells were infected with VSVΔG*-Lloviu or VSVΔG*-Zaire in the presence of mouse antisera specific to the respective viruses (Fig. 7). We confirmed the ADE activity of the anti-Zaire serum as indicated by markedly enhanced infectivities of VSVΔG*-Zaire at the serum dilutions of 1:10 and 1:100 (Fig 7A). By contrast, only minimal ADE activity

was seen in the anti-LLOV serum, although similar amounts of specific IgG antibodies were detected in both antisera by ELISA (Fig. 4). Consistent with the absence of cross-reactive IgG in ELISA, little cross-ADE activity was observed in the ADE assay.

Cellular tropism of LLOV GP. To estimate the GP-dependent tropism that is likely reflected by the prevalence of LLOV receptors, we infected various cell lines of different animal origins (Table 1) with pseudotyped VSVs and their infectivities were compared (Fig. 8A). VSVΔG*-Lloviu infected cells that were derived from the human, African green monkey, pig, dog, and bat in a similar manner to VSVΔG*-Zaire and VSVΔG*-Reston (Fig. 8A). VSVΔG*-Angola and VSVΔG*-Musoke had higher IUs in these cells than VSVΔG*-Lloviu, VSVΔG*-Zaire, and VSVΔG*-Reston, except in a cell line from the Yaeyama flying fox (*Pteropus dasymallus yayeyamae*; FBKT1) (Fig. 8A). Since some species of bats are suspected to be natural reservoirs of filoviruses (33-37), we focused on these bat cells and relative infectivities were determined (Fig. 8B). Interestingly, VSVΔG*-Lloviu infected IndFSPT1 and SuBK12-08 more efficiently than the other viruses tested.

DISCUSSION

This study provides fundamental information on the properties of LLOV GP. The RNA editing that is required to produce the full length transmembrane GP is a common characteristic of viruses belonging to the genus *Ebolavirus* (21, 22). The presence of the editing site in the LLOV GP gene supports the notion that this virus is more related to ebolaviruses than marburgviruses. Phylogenetic analyses also suggested that LLOV might have the same ancestor as ebolaviruses (4). On the other hand, LLOV GP has MLR and a furin cleavage site, both of which are common features shared by previously known filoviruses (38, 39). By TEM and SEM of VLPs, we further found morphological similarity between LLOV and other filoviruses, which have numerous GP spikes located on the filamentous VLP surface. Viral entry assays with chemical inhibitors also suggest that LLOV GP, as well as GPs of the other filoviruses, requires low endosomal pH and proteolytic processing for virus entry into cells. Taken together, these results indicate that the structure and function of LLOV GP are primarily similar to those of the other filovirus GPs.

However, the requirement of cathepsin L might be controversial since a high concentration ($> 1 \mu\text{M}$) of FY-dmk was suggested to inhibit not only cathepsin L but also cathepsins B and likely other endosomal cysteine proteases (40). FY-dmk at the lowest concentration tested in this study ($0.625 \mu\text{M}$) did not reduce infectivities of neither VSV ΔG^* -Lloviu, VSV ΔG^* -Zaire, nor VSV ΔG^* -Angola, suggesting that cathepsin L is not essential for LLOV infection similarly to the other filoviruses (40, 41). It was also shown that Cathepsin B and Cathepsin L activities are not required for EBOV replication in a mouse model (41). Thus, further studies are needed to clarify the

in vivo importance of the GP cleavage by cathepsins and some other host proteases for filovirus infection.

Hepatocytes, dendritic cells, monocytes, and macrophages, all of which express cell surface C-type lectins, are thought to be the preferred target cells of filoviruses and increased infection of these cells might be directly involved in the pathogenesis of filovirus infection (11, 19, 42-44). We demonstrated that LLOV GP utilized human C-type lectins, hMGL and DC-SIGN, most likely as attachment factors, as reported with the other filovirus GPs (13, 19, 20, 45). These C-type lectins have different glycan specificities (i.e. hMGL and DC-SIGN preferentially react with O- and high-mannose type N-glycans, respectively) (46-48). Like EBOV and RESTV GPs, LLOV GP showed greater preference for DC-SIGN than hMGL compared with MARV GPs, suggesting that LLOV GP, particularly its MLR, might have a carbohydrate structure comparable to that of ebolaviruses. Our data suggest that LLOV GP has a tropism to cells expressing C-type lectins and might potentially infect human immune cells such as dendritic cells and macrophages.

Like C-type lectins, ADE antibodies mostly recognize epitopes on MLR of filovirus GPs, leading to enhanced infectivity (11, 13, 42). Thus, MLR is thought to play important roles for these two attachment functions of GP. In addition to the primary structure of MLR (i.e., the presence of different epitopes and sugar chains among filoviruses), the GP2 region seems to have key amino acid residues (e.g., the amino acid at position 547 in MARV GP) contributing to the efficiency of ADE- and C-type lectin-mediated entry (15, 20). Although detailed functional mapping and structural analysis are still needed, our data indicate that the overall properties of LLOV

GP for ADE- and C-type lectin-mediated entry are comparable to those of RESTV GP rather than highly virulent EBOV and MARV (strain Angola) (11, 13, 42).

VSVΔG*-Lloviu infected all cell lines tested as well as VSVΔG*-Zaire and VSVΔG*-Reston (Fig. 5A). Recently, RESTV was detected in pigs in the Philippines and China, and more recent studies have revealed that EBOV causes respiratory disease in pigs (49-53), suggesting a potential role of this animal in filovirus ecology. In this study, VSVΔG*-Lloviu infected pig cells (SK-L cells) in a similar manner to VSVΔG*-Zaire and VSVΔG*-Reston. Although GP is not the only determinant controlling filovirus pathogenicity, our data suggest that LLOV, at least, meets the minimum requirements to infect pig cells.

Interestingly, neither VSVΔG*-Angola nor VSVΔG*-Musoke infected FBKT1 cells derived from the Yaeyama flying fox (*Pteropus dasymallus yayeyamae*), although the infectivities of these viruses in the other cell lines tested were uniformly higher than those of the other viruses (Fig.5A). This finding suggests the existence of cellular receptors/coreceptors that interacts with EBOV, RESTV, and LLOV GPs but not MARV GP. Although several cellular molecules were reported to be involved in filovirus entry (e.g., T-cell immunoglobulin domain and mucin domain 1, Tyro 3 family, C-type lectin, or Niemann-Pick C1) (11-13, 42-47, 54-57), there is only limited information on these molecules in bats. It would be of interest to clarify whether these cellular molecules play critical roles in tissue tropism and/or host range restriction of filoviruses. Alternatively, there might be a new receptor of LLOV in bats.

VSVΔG*-Lloviu infected SuBK12-08 and IndFSPT1 more efficiently than the other viruses (Fig. 5B). It should be noted that SuBK12-08 was derived from the same insectivorous bat species, Schreiber's bat (*Miniopterus schreibersii*), in which LLOV

was first detected in Europe. However, since LLOV likely causes lethal infection of Schreiber's bat, this bat species may not serve as the natural host that can maintain this virus in nature. On the other hand, IndFSPT1 was derived from fruit bats. Considering that some species of fruit bats are suspected to be natural hosts of filoviruses (33, 58), strong tropism to this fruit bat species may suggest that fruit bats also play some roles in the ecology of LLOV.

While LLOV seems to be highly pathogenic for some species of bats (e.g., Schreiber's bat), its ability to infect human and nonhuman primates and the pathogenic potential for these hosts can only be hypothesized, since infectious LLOV has never been isolated. In this study, we used a replication-incompetent VSV pseudotype system that enabled us to investigate the cellular tropism mediated by simple interaction between LLOV GP and its cellular ligands. Although a reverse genetics approach and *in vivo* experiments for infectious LLOV are needed to provide direct evidence of the viral pathogenicity and host specificity, our data suggest that the overall properties of LLOV GP are similar to those of the other filoviruses, and that LLOV has the potential, at least from the aspect of GP-receptor/coreceptor interaction, to infect many mammalian cells, including those of the human, monkey, and pig, with preferential tropism for some bat cells.

ACKNOWLEDGMENTS

We thank Mari Ishijima for technical assistance and Kim Barrymore for editing the manuscript. This work was supported by KAKENHI, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Japan Society for the Promotion of Science (JSPS), Japan, and partly by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and the Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA) within the framework of the Science and Technology Research Partnership for Sustainable Development (SATREPS). Funding was also provided by the Program for Leading Graduate Schools from MEXT, Japan.

REFERENCES

1. **Feldmann H, Geisbert TW.** 2011. Ebola haemorrhagic fever. *Lancet* **377**:849-862.
2. **Fisher-Hoch SP, McCormick JB.** 1999. Experimental filovirus infections. *Curr. Top. Microbiol. Immunol.* **235**:117-143.
3. **Geisbert TW, Hensley LE.** 2004. Ebola virus: new insights into disease aetiopathology and possible therapeutic interventions. *Expert. Rev. Mol. Med.* **6**:1-24.
4. **Negredo A, Palacios G, Vázquez-Morón S, González F, Dopazo H, Molero F, Juste J, Quetglas J, Savji N, de la Cruz Martínez M, Herrera JE, Pizarro M, Hutchison SK, Echevarría JE, Lipkin WI, Tenorio A.** 2011. Discovery of an ebolavirus-like filovirus in europe. *PLoS Pathog.* **7**:e1002304.
5. **Elliott LH, Kiley MP, McCormick JB.** 1985. Descriptive analysis of Ebola virus proteins. *Virology* **147**:169-176.
6. **Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y.** 1997. A system for functional analysis of Ebola virus glycoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **94**:14764-14769.
7. **Wool-Lewis RJ, Bates P.** 1998. Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. *J. Virol.* **72**:3155-3160.
8. **Manicassamy B, Wang J, Rumschlag E, Tymen S, Volchkova V, Volchkov V, Rong L.** 2007. Characterization of Marburg virus glycoprotein in viral entry. *Virology* **358**:79-88.
9. **Geyer H, Will C, Feldmann H, Klenk HD, Geyer R.** 1992. Carbohydrate structure of Marburg virus glycoprotein. *Glycobiology* **2**:299-312.

- 448 10. **Feldmann H, Nichol ST, Klenk HD, Peters CJ, Sanchez A.** 1994.
449 Characterization of filoviruses based on differences in structure and antigenicity of
450 the virion glycoprotein. *Virology* **199**:469-473.
- 451 11. **Alvarez CP, Lasala F, Carrillo J, Muñiz O, Corbí AL, Delgado R.** 2002. C-type
452 lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in
453 trans. *J Virol* **76**:6841-6844.
- 454 12. **Baribaud F, Pöhlmann S, Leslie G, Mortari F, Doms RW.** 2002. Quantitative
455 expression and virus transmission analysis of DC-SIGN on monocyte-derived
456 dendritic cells. *J. Virol.* **76**:9135-9142.
- 457 13. **Lin G, Simmons G, Pöhlmann S, Baribaud F, Ni H, Leslie GJ, Haggarty BS,**
458 **Bates P, Weissman D, Hoxie JA, Doms RW.** 2003. Differential N-linked
459 glycosylation of human immunodeficiency virus and Ebola virus envelope
460 glycoproteins modulates interactions with DC-SIGN and DC-SIGNR. *J. Virol.*
461 **77**:1337-1346.
- 462 14. **Takada A, Ebihara H, Feldmann H, Geisbert TW, Kawaoka Y.** 2007. Epitopes
463 required for antibody-dependent enhancement of Ebola virus infection. *J. Infect.*
464 *Dis.* **196 Suppl 2**:S347-356.
- 465 15. **Nakayama E, Tomabechi D, Matsuno K, Kishida N, Yoshida R, Feldmann H,**
466 **Takada A.** 2011. Antibody-dependent enhancement of Marburg virus infection. *J.*
467 *Infect. Dis.* **204 Suppl 3**:S978-985.
- 468 16. **Feldmann H, Mühlberger E, Randolph A, Will C, Kiley MP, Sanchez A, Klenk**
469 **HD.** 1992. Marburg virus, a filovirus: messenger RNAs, gene order, and regulatory
470 elements of the replication cycle. *Virus Res.* **24**:1-19.

- 471 17. **Sakoda Y, Fukusho A.** 1998. Establishment and characterization of a porcine
472 kidney cell line, FS-L3, which forms unique multicellular domes in serum-free
473 culture. *In vitro cellular & developmental biology. Animal* **34**:53-57.
- 474 18. **Maeda K, Hondo E, Terakawa J, Kiso Y, Nakaichi N, Endoh D, Sakai K,**
475 **Morikawa S, Mizutani T.** 2008. Isolation of novel adenovirus from fruit bat
476 (*Pteropus dasymallus yayeyamae*). *Emerg. Infect. Dis.* **14**:347-349.
- 477 19. **Takada A, Fujioka K, Tsuiji M, Morikawa A, Higashi N, Ebihara H, Kobasa**
478 **D, Feldmann H, Irimura T, Kawaoka Y.** 2004. Human macrophage C-type lectin
479 specific for galactose and N-acetylgalactosamine promotes filovirus entry. *J. Virol.*
480 **78**:2943-2947.
- 481 20. **Matsuno K, Kishida N, Usami K, Igarashi M, Yoshida R, Nakayama E,**
482 **Shimojima M, Feldmann H, Irimura T, Kawaoka Y, Takada A.** 2010. Different
483 potential of C-type lectin-mediated entry between Marburg virus strains. *J. Virol.*
484 **84**:5140-5147.
- 485 21. **Sanchez A, Trappier SG, Mahy BW, Peters CJ, Nichol ST.** 1996. The virion
486 glycoproteins of Ebola viruses are encoded in two reading frames and are expressed
487 through transcriptional editing. *Proc. Natl. Acad. Sci. U. S. A.* **93**:3602-3607.
- 488 22. **Volchkov VE, Becker S, Volchkova VA, Ternovoj VA, Kotov AN, Netesov SV,**
489 **Klenk HD.** 1995. GP mRNA of Ebola virus is edited by the Ebola virus
490 polymerase and by T7 and vaccinia virus polymerases. *Virology* **214**:421-430.
- 491 23. **Niwa H, Yamamura K, Miyazaki J.** 1991. Efficient selection for high-expression
492 transfectants with a novel eukaryotic vector. *Gene* **108**:193-199.
- 493 24. **Changula K, Yoshida R, Noyori O, Marzi A, Miyamoto H, Ishijima M,**
494 **Yokoyama A, Kajihara M, Feldmann H, Mweene AS, Takada A.** 2013.

Mapping of conserved and species-specific antibody epitopes on the Ebola virus nucleoprotein. *Virus Res.*, in press.

25. **Nakayama E, Yokoyama A, Miyamoto H, Igarashi M, Kishida N, Matsuno K, Marzi A, Feldmann H, Ito K, Saijo M, Takada A.** 2010. Enzyme-linked immunosorbent assay for detection of filovirus species-specific antibodies. *Clin. Vaccine Immunol.* **17**:1723-1728.

26. **Noda T, Sagara H, Suzuki E, Takada A, Kida H, Kawaoka Y.** 2002. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. *J. Virol.* **76**:4855-4865.

27. **Noda T, Ebihara H, Muramoto Y, Fujii K, Takada A, Sagara H, Kim JH, Kida H, Feldmann H, Kawaoka Y.** 2006. Assembly and budding of Ebolavirus. *PLoS Pathog.* **2**:e99.

28. **Johnson RF, Bell P, Harty RN.** 2006. Effect of Ebola virus proteins GP, NP and VP35 on VP40 VLP morphology. *Virol. J.* **3**:31.

29. **Geisbert TW, Jahrling PB.** 1995. Differentiation of filoviruses by electron microscopy. *Virus Res.* **39**:129-150.

30. **Chandran K, Sullivan NJ, Felbor U, Whelan SP, Cunningham JM.** 2005. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. *Science* **308**:1643-1645.

31. **Takada A, Watanabe S, Okazaki K, Kida H, Kawaoka Y.** 2001. Infectivity-enhancing antibodies to Ebola virus glycoprotein. *J. Virol.* **75**:2324-2330.

- 517 32. **Takada A, Kawaoka Y.** 2003. Antibody-dependent enhancement of viral
518 infection: molecular mechanisms and in vivo implications. *Rev. Med. Virol.*
519 **13**:387-398.
- 520 33. **Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A,**
521 **Swanepoel R, Paddock CD, Balinandi S, Khristova ML, Formenty PB,**
522 **Albarino CG, Miller DM, Reed ZD, Kayiwa JT, Mills JN, Cannon DL, Greer**
523 **PW, Byaruhanga E, Farnon EC, Atimnedi P, Okware S, Katongole-Mbidde E,**
524 **Downing R, Tappero JW, Zaki SR, Ksiazek TG, Nichol ST, Rollin PE.** 2009.
525 Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS*
526 *Pathog.* **5**:e1000536.
- 527 34. **Amman BR, Carroll SA, Reed ZD, Sealy TK, Balinandi S, Swanepoel R,**
528 **Kemp A, Erickson BR, Comer JA, Campbell S, Cannon DL, Khristova ML,**
529 **Atimnedi P, Paddock CD, Crockett RJ, Flietstra TD, Warfield KL, Unfer R,**
530 **Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Rollin PE, Ksiazek**
531 **TG, Nichol ST, Towner JS.** 2012. Seasonal Pulses of Marburg Virus Circulation
532 in Juvenile *Rousettus aegyptiacus* Bats Coincide with Periods of Increased Risk of
533 Human Infection. *PLoS Pathog.* **8**:e1002877.
- 534 35. **Towner JS, Pourrut X, Albariño CG, Nkogue CN, Bird BH, Grard G, Ksiazek**
535 **TG, Gonzalez JP, Nichol ST, Leroy EM.** 2007. Marburg virus infection detected
536 in a common African bat. *PloS One* **2**:e764.
- 537 36. **Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P,**
538 **Délicat A, Paweska JT, Gonzalez JP, Swanepoel R.** 2005. Fruit bats as reservoirs
539 of Ebola virus. *Nature* **438**:575-576.

- 540 37. **Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP, Leroy E.**
541 2009. Large serological survey showing cocirculation of Ebola and Marburg
542 viruses in Gabonese bat populations, and a high seroprevalence of both viruses in
543 *Rousettus aegyptiacus*. *BMC Infect. Dis.* **9**:159.
- 544 38. **Volchkov VE, Feldmann H, Volchkova VA, Klenk HD.** 1998. Processing of the
545 Ebola virus glycoprotein by the proprotein convertase furin. *Proc. Natl. Acad. Sci.*
546 *U. S. A.* **95**:5762-5767.
- 547 39. **Volchkov VE, Volchkova VA, Ströher U, Becker S, Dolnik O, Cieplik M,**
548 **Garten W, Klenk HD, Feldmann H.** 2000. Proteolytic processing of Marburg
549 virus glycoprotein. *Virology* **268**:1-6.
- 550 40. **Misasi, J., Chandran, K., Yang, J. Y., Considine, B., Filone, C. M., Cote, M.,**
551 **Sullivan, N., Fabozzi, G., Hensley, L.** 2012. Filoviruses require endosomal
552 cysteine proteases for entry but exhibit distinct protease preferences. *J Virol.*
553 **86**:3284-3292
- 554 41. **Marzi, A., Reinheckel, T., Feldmann, H.** 2012. Cathepsin B & L are not required
555 for ebola virus replication. *PLoS Negl Trop Dis* **6**:e1923.
- 556 42. **Simmons G, Reeves JD, Grogan CC, Vandenberghe LH, Baribaud F,**
557 **Whitbeck JC, Burke E, Buchmeier MJ, Soilleux EJ, Riley JL, Doms RW,**
558 **Bates P, Pöhlmann S.** 2003. DC-SIGN and DC-SIGNR bind ebola glycoproteins
559 and enhance infection of macrophages and endothelial cells. *Virology* **305**:115-123.
- 560 43. **Dominguez-Soto A, Aragoneses-Fenoll L, Martin-Gayo E, Martinez-Prats L,**
561 **Colmenares M, Naranjo-Gomez M, Borrás FE, Munoz P, Zubiaur M, Toribio**
562 **ML, Delgado R, Corbi AL.** 2007. The DC-SIGN-related lectin LSECtin mediates

antigen capture and pathogen binding by human myeloid cells. *Blood* **109**:5337-5345.

44. **Powlesland AS, Fisch T, Taylor ME, Smith DF, Tissot B, Dell A, Pöhlmann S, Drickamer K.** 2008. A novel mechanism for LSECtin binding to Ebola virus surface glycoprotein through truncated glycans. *The Journal of biological chemistry* **283**:593-602.

45. **Matsuno K, Nakayama E, Noyori O, Marzi A, Ebihara H, Irimura T, Feldmann H, Takada A.** 2010. C-type lectins do not act as functional receptors for filovirus entry into cells *Biochem. Biophys. Res. Commun.* **403**:144-148.

46. **Marzi A, Möller P, Hanna SL, Harrer T, Eisemann J, Steinkasserer A, Becker S, Baribaud F, Pöhlmann S.** 2007. Analysis of the interaction of Ebola virus glycoprotein with DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) and its homologue DC-SIGNR. *J. Infect. Dis.* 196 Suppl **2**:S237-246.

47. **Feinberg H, Mitchell DA, Drickamer K, Weis WI.** 2001. Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. *Science* **294**:2163-2166.

48. **Higashi N, Fujioka K, Denda-Nagai K, Hashimoto S, Nagai S, Sato T, Fujita Y, Morikawa A, Tsuiji M, Miyata-Takeuchi M, Sano Y, Suzuki N, Yamamoto K, Matsushima K, Irimura T.** 2002. The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells. *J. Biol. Chem.* **277**:20686-20693.

- 585 49. **Suzuki N, Yamamoto K, Toyoshima S, Osawa T, Irimura T.** 1996. Molecular
586 cloning and expression of cDNA encoding human macrophage C-type lectin. Its
587 unique carbohydrate binding specificity for Tn antigen. *J. Immunol.* **156**:128-135.
- 588 50. **Barrette RW, Metwally SA, Rowland JM, Xu L, Zaki SR, Nichol ST, Rollin**
589 **PE, Towner JS, Shieh WJ, Batten B, Sealy TK, Carrillo C, Moran KE, Bracht**
590 **AJ, Mayr GA, Sirios-Cruz M, Catbagan DP, Lautner EA, Ksiazek TG, White**
591 **WR, McIntosh MT.** 2009. Discovery of swine as a host for the Reston ebolavirus.
592 *Science* **325**:204-206.
- 593 51. **Marsh GA, Haining J, Robinson R, Foord A, Yamada M, Barr JA, Payne J,**
594 **White J, Yu M, Bingham J, Rollin PE, Nichol ST, Wang LF, Middleton D.**
595 2011. Ebola Reston virus infection of pigs: clinical significance and transmission
596 potential. *J. Infect. Dis.* 204 Suppl 3:S804-809.
- 597 52. **Pan Y, Zhang W, Cui L, Hua X, Wang M, Zeng Q.** 2012. Reston virus in
598 domestic pigs in China. *Arch. Virol.*, in press.
- 599 53. **Nfon CK, Leung A, Smith G, Embury-Hyatt C, Kobinger G, Weingartl HM.**
600 2013. Immunopathogenesis of severe acute respiratory disease in Zaire
601 ebolavirus-infected pigs. *PloS One* **8**:e61904.
- 602 54. **Kondratowicz AS, Lennemann NJ, Sinn PL, Davey RA, Hunt CL,**
603 **Moller-Tank S, Meyerholz DK, Rennert P, Mullins RF, Brindley M,**
604 **Sandersfeld LM, Quinn K, Weller M, McCray PB Jr, Chiorini J, Maury W.**
605 2011. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire
606 Ebolavirus and Lake Victoria Marburgvirus. *Proc. Natl. Acad. Sci. U. S. A.*
607 **108**:8426-8431.

- 608 55. **Shimajima M, Takada A, Ebihara H, Neumann G, Fujioka K, Irimura T,**
609 **Jones S, Feldmann H, Kawaoka Y.** 2006. Tyro3 family-mediated cell entry of
610 Ebola and Marburg viruses. *J. Virol.* **80**:10109-10116.
- 611 56. **Côté M, Misasi J, Ren T, Bruchez A, Lee K, Filone CM, Hensley L, Li Q, Ory**
612 **D, Chandran K, Cunningham J.** 2011. Small molecule inhibitors reveal
613 Niemann-Pick C1 is essential for Ebola virus infection. *Nature* **477**:344-348.
- 614 57. **Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N,**
615 **Kuehne AI, Kranzusch PJ, Griffin AM, Ruthel G, Dal Cin P, Dye JM, Whelan**
616 **SP, Chandran K, Brummelkamp TR.** 2011. Ebola virus entry requires the
617 cholesterol transporter Niemann-Pick C1. *Nature* **477**:340-343.
- 618 58. **Swanepoel R, Smit SB, Rollin PE, Formenty P, Leman PA, Kemp A, Burt FJ,**
619 **Grobbelaar AA, Croft J, Bausch DG, Zeller H, Leirs H, Braack LE, Libande**
620 **ML, Zaki S, Nichol ST, Ksiazek TG, Paweska JT.** 2007. Studies of reservoir
621 hosts for Marburg virus. *Emerg. Infect. Dis.* **13**:1847-1851.

FIGURE LEGENDS

FIG. 1 Primary structure of LLOV envelope GP. Nucleotide and deduced amino acid sequences of LLOV GP (A). Deduced amino acid sequences are shown in bold letters. Blue, green, and yellow lines represent the signal peptide, MLR, and transmembrane domain predicted by GENETYX Ver.10, NetOGlyc, and TopPred 0.01, respectively. Purple and red letters represent the putative editing and cleavage sites, respectively. Comparison of EBOV and LLOV MLRs (B). Potential O-glycosylation sites were predicted by NetOGlyc, and MLRs were defined as the regions between the first and last amino acid residues showing the score over the threshold (0.5). Arrows indicate the cleavage sites. Western blotting of filovirus GPs (C). Proteins in the lysate of HEK293T cells transfected with the plasmid expressing LLOV GP (lanes 1 and 2), EBOV GP (lanes 3 and 4), MARV GP (lanes 5 and 6), or empty vector (lanes 7 and 8) were separated by SDS-PAGE under non-reducing (lanes 1, 3, 5, and 7) or reducing (lanes 2, 4, 6, and 8) conditions.

FIG. 2 TEM of filovirus VLPs. Purified VLPs produced from 293T cells transfected with plasmids expressing LLOV (A, B, E, and F), EBOV (Zaire) (C and G), and MARV (Angola) (D and H) proteins were fixed and stained as described in Materials and Methods. For immuno-TEM (B and F), an anti-LLOV GP monoclonal antibody was used. Scale bars represent 500 nm (A to D) and 200 nm (E to H). Arrowheads indicate gold particles.

FIG. 3 SEM of LLOV VLPs. HEK293T cells transfected with pCAGGS expressing LLOV GP, VP40, and NP (A and B) or pCAGGS alone (C and D) were fixed at 48 hours after transfection. Samples were observed with an S-4700 scanning electron microscope (Hitachi). Scale bars represent 5 μm (A and C) and 2 μm (B and D).

FIG. 4 Cross-reactivities of anti-GP sera among filoviruses in ELISA. Ten-fold serial dilutions of mouse antisera to EBOV (anti-Zaire), SUDV (anti-Sudan), TAFV (anti-Tai forest), BDBV (anti-Bundibugyo), RESTV (anti-Reston), LLOV (anti-Lloviu), and MARV (anti-Marburg) were tested for IgG reactivities to LLOV (A), EBOV (B), SUDV (C), TAFV (D), BDBV (E), RESTV (F), and MARV (G) GP antigens. Three mice were used for each virus and averages and standard deviations are shown.

FIG. 5 Effects of chemical inhibitors on infectivities of pseudotyped VSVs. Vero E6 cells were pretreated with ammonium chloride (AMC; A), monensin (MON; B), and cathepsin B and L inhibitors (Cat B; C, and Cat L; D) for 30 min at 37°C. The treated cells were then infected with VSV Δ G*-Zaire (Zaire), VSV Δ G*-Angola (Angola), VSV Δ G*-Lloviu (Lloviu), and VSV Δ G*-G (VSV) appropriately diluted to yield 200–2000 IUs/ 10^6 cells. At 20 hours post-inoculation, GFP-positive cells were counted using an IN Cell Analyzer 2000 (GE Healthcare). The percentages of infectivity were determined by setting the number of the untreated cells to 100%. Each experiment was performed three times, and averages and standard deviations are shown.

FIG. 6 Infectivities of pseudotyped VSVs in C-type lectin-expressing cells. K562 cells expressing hMGL or DC-SIGN were infected with VSV Δ G*-Zaire,

VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke. The infectivity of each pseudotyped VSV on K562 clones was determined by counting the number of GFP-positive cells using flow cytometry, and the percentages of infectivity in K562-hMGL and -DC-SIGN were determined by setting the number of the infected K562 to 100%. Each experiment was performed three times, and averages and standard deviations are shown. Statistical significance of the differences was determined by Student's t-test (see P values in the text).

FIG. 7 ADE activities of antisera to EBOV and LLOV GPs. Ten-fold serially diluted mouse anti-EBOV (A) and anti-LLOV (B) sera were mixed with equal volumes of the VSVΔG*-Zaire and VSVΔG*-Lloviu. Relative infectivity was determined by setting the number of infected K562 cells without antisera to 100%. Each experiment was performed three times, and averages and standard deviations are shown.

FIG. 8 Infectivities of pseudotyped VSVs in mammalian cell lines. VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke were inoculated into several mammalian cell lines. Infectious units (IUs) of each virus in different cell lines were determined by counting the number of GFP-expressing cells and each IU was then standardized based on 10^6 copies of the VSV genome determined by real-time RT-PCR (A). Relative infectivities in bat cell lines are given by setting each IU in Vero E6 to 1.0 (i.e., [IU in bat cells]/[IU in Vero E6 cells] (B). Each experiment was performed three times, and averages and standard deviations are shown. Infectivities of VSVΔG*-Angola, and VSVΔG*-Musoke were under the limit of detection (*).

1 **TABLE 1** Origins of cell lines used in this study

Cell line	Species	Zoological name	Organ
Vero E6	African green monkey	<i>Chlorocebus</i> sp.	Kidney
HEK293	Human	<i>Homo sapiens</i>	Kidney
SK-L	Pig	<i>Sus scrofa domesticus</i>	Kidney
MDCK	Dog	<i>Canis lupus familiaris</i>	Kidney
BKT1	Greater horseshoe bat ^a	<i>Rhinolophus ferrumequinum</i>	Kidney
FBKT1	Yaeyama flying fox ^b	<i>Pteropus dasymallus yayeyamae</i>	Kidney
YubFKT1	Eastern bent-winged bat ^c	<i>Miniopterus fuliginosus</i>	Kidney
IndFSPT1	Indian flying fox ^d	<i>Pteropus giganteus</i>	Spleen
DemKT1	Leschenault's rousette ^e	<i>Rousettus leschenaulti</i>	Kidney
ZFB11-97	Gambian epauletted fruit bat ^f	<i>Epomophorus gambianus</i>	Kidney
SuBK12-08	Schreiber's bat ^g	<i>Miniopterus schreibersii</i>	Kidney

2 ^a Nucleotide sequence identity is 98% (manuscript in preparation).

3 ^b Previously described (18).

4 ^c Nucleotide sequence identity is 99%.

5 ^d Nucleotide sequence identity is 98%.

6 ^e Nucleotide sequence identity is 100%.

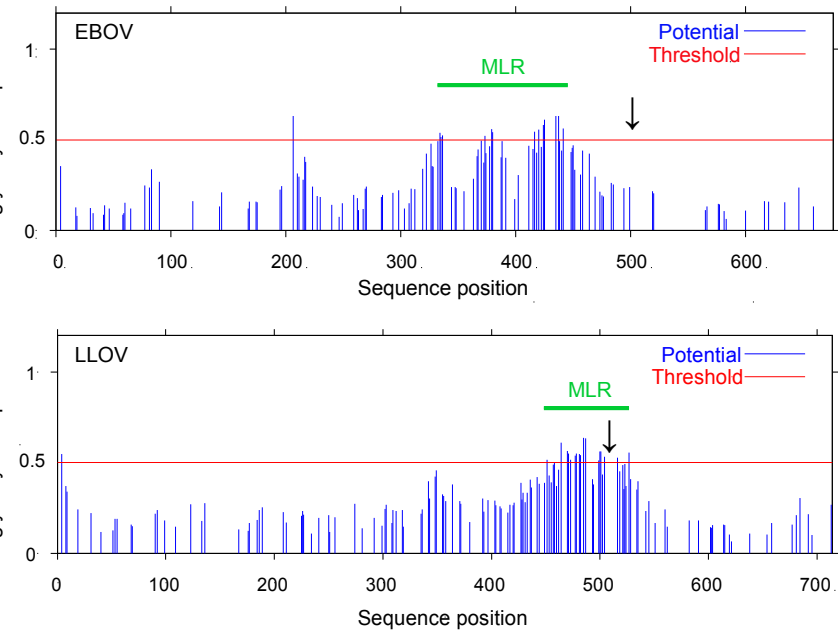
7 ^f Nucleotide sequence identity is 89%.

8 ^g Nucleotide sequence identity is 98%.

A

M V P T Y P Y S S L L D W R P P P N T L P W I L N L V V F Y T I A W L P G G V S 40
ATGGTGCCCA CCTACCCGTA CAGCAGCCTA TTAGATTGGA GACCACCACC AAACACCCTA CCATGGATCC TCAACCTTGT GGTCTTTTAT ACCATAGCCT GGCTGCCCGG GGGAGTCTCA 120
G I P L G L L G N N S I T Q T V V D N V V C K E H L A T T D Q L Q A I G L G L E 80
GGAATTCAC TCGGTTTGT GGGAAACAAC AGCATCACCC AAAGTGTCTG GGACAATGTA GTGTGCAAGG AACACCTTGC CACAACAGAT CAGCTACAGG CTATTGGATT GGGACTAGAG 240
G L G E H A D L P T A T K R W G F R S D V I P K I V G Y T A G E W V E N C Y N L 120
GGGCTTGGTG AACATGCTGA CCTCCCGACT GCCACCAAGC GATGGGGTTT TCGATCTGAT GTCATCCCAA AAATCGTGGG ATACACCGCT GGGAATGGG TGGAAACTG CTACAATCTT 360
E I T K K D G H P C L P S P P T G L L G Y P R C R Y V H R A K G A G P C P G G N 160
GAAATCACCA AGAAAGATGG TCATCCTTGC CTCGCCAGCC CGCCAAGTGG CTTACTTGGC TATCCCGGAT GCCGCTATGT CCACAGAGCC AAAGGAGCAG GCCCTTGGCC AGGTGGGAAT 480
A F H K H G S F F L Y H G M A S T V I Y H G V T F T E G T I A F L I V P K D A P 200
GCTTTCACA AACATGGTTC TTTCTTTCTG TACCACGGTA TGGCTTCTAC AGTAATTTAT CATGGTGTA CTTTACGGA AGGCACAATT GGTTCCTAA TTGTCCCGAA GGATGCACCC 600
R L K A G L G T G F S H Q A E N Q N P N N Q F R T T T L D Y D V M S P W M D N A 240
CGTCTCAAG CAGGGCTTGG AACAGGATTC AGTCATCAAG CAGAGAACCA AAACCCAAC AACCAATTC GAACAACAAC TTTAGATTAT GATGTAATGA GTCCTTGGAT GGACAATGCT 720
T F F F R A R E D T S M L I Q T R Y P P A N L E L V Q E R L A N L T C G D Q A D P 280
ACCTTCACA TTCGAGCGAG GGAAGACACA TCAAGATGAG GTACCTTCCA GTCAATCTAG AGCTTGTGCA AGAAACGATTG AGGAGATCTA CGGAGATCA AGCTATGCCA 840
S K M E E I V A E V L T L E L G D W S G W T T K K N R S T N H T A K K P F T S I 320
TCAAAGATGG AAGAGATTGT CGCTGAGGTT TTGACATTGG AGCTCGGTGA TTGGTCCGGT TGGACAACCTG AAAAAACCG CAGTACAAAC CATACGGCTA AGAAACCTT CACCAAGCATC 960
W F N Q G Q D W P E A H D G S S G V H P P T S F C C W T T L P L E H S S N S G A 360
TGTTCAACC AAGGACAAGA CTGGCCAGAA GCCCATGAGC GATCATCAGG AGTTCATCCT CCAACCTCAT TCTGCTGTTG GACAACCGTG CCTCTGGAAC ATTCTTCGAA CTCCGGGGCG 1080
E P C T K A P A G N T T N N V H H C C S W V R I Q A V H P G N T S G E I S M P L 400
GAACCTGCA CGAAGGCACC GGCGGGAAAC ACCACCAACA ATGTCCATCA CTGCTGCTCC TGGGTCAGGA TACAAGCGT ACATCCAGGC AATACCTCTG GTGAAATTC GATGCCATTG 1200
G G S S A C V S S I P L L G S V S N N S S I Q E L E T S S K S A T E L T T P I N 440
GGAGGGTCTT CGGCATGTGT GTCGTCGATA CCCCTCCTGG GTTCAGTGAG CAACAATAGT TCAATACAGG AGCTTGAGAC TTCATCTAAA AGTGCAACAG AATTGACAAC TCCCATCAAT 1320
H S Q S L Q L A S V T N T P T P T T Q S K S W T V D Y N N T T P T M D P T T I L 480
CACTCCCAAT CACTACAGCT CGCATCCGTC ACAAACACCC CCACACCGAG AACACAGTCC AAGTCCTGGA CAGTTGAGTA CAACAACACA ACGCCAACCA TGGATCCCAAC AACATACTG 1440
T T P D T A T I P P N N S S D H N A T T T S K T R R R R Q V N P V P P T I T Q Q 520
ACGACACCCG ACACCGCAAC CATTCCCTCT AACAACCTAT CTGATCACAA CGCCACAACA ACAAGCAAAA CAAGACGAAG GAGACAGGTC AACCCAGTGC CCCCAACGAT CACCCAACAA 1560
T S T S I N T S H H P N M T T Q L A R H P S V Q T R M Q N P S C N P N L R Y W T 560
ACCTCTACAA GCATCAATAC CTCGCCACAC CCCAATATGA CAACACAGTT AGCAAGACAT CCGAGTGTGC AAACAAGGAT GCAAAACCCC AGCTGTAATC CCAACCTTAG ATACTGGACA 1680
S R E M S N A G G L A W I P W I G P G I E G G I T D G I M E H Q N T I V C Q L R 600
AGCCGGGAGA TGAGTAATGC TGGGGGGCTT GCATGGATTC CATGGATTGG ACCAGGGATT GAGGGAGGGA TCACAGACGG GATAATGGAG CATCAGAACA CAATTGTCTG TCAGTTACGG 1800
E L A N T T T K A L Q L F L R A T T E L R T Y S I L N R H A I D F L L Q R W G G 640
GAGTCTCGGA ACACCACTAC TAAAGCCTA CAGCTTTTCC TCCGGGCTAC CACTGAGCTC CGAACCTACT CTATCCTCAA CCGCCATGCG ATTGACTTTC TACTACAGCG TTGGGGTGGT 1920
T C R I L G P N C C I E P H D W S A N I T A E I N H I R E D I L N H H E I Q P S 680
ACCTGCAGAA TCCTTGGCCC AAAGTGTCTG ATCGAACCTC ATGATTGGTC TGCCAACATT ACGGCTGAGA TAAATCATAT TAGAGAAGAT ATCCTGAAAC ATCATGAGAT CCAACCTTCT 2040
Q D P S F W T G W Q Q W I P T G A S A L G I I L A I L A L I C L C R I T R 717 amino acids
CAAGACCCCT CCTTTTGGAC TGGATGGCAA CAGTGGATCC CAACAGGAGC CAGTGTCTCT GGAATCATCC TGGAATATT AGCCTTGATT TGTCTGTGCA GAATAACAG A 2151 nucleotides

B



C

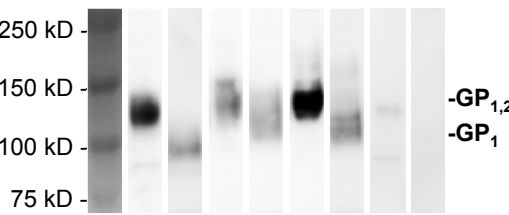


Fig. 1 Maruyama *et al.*

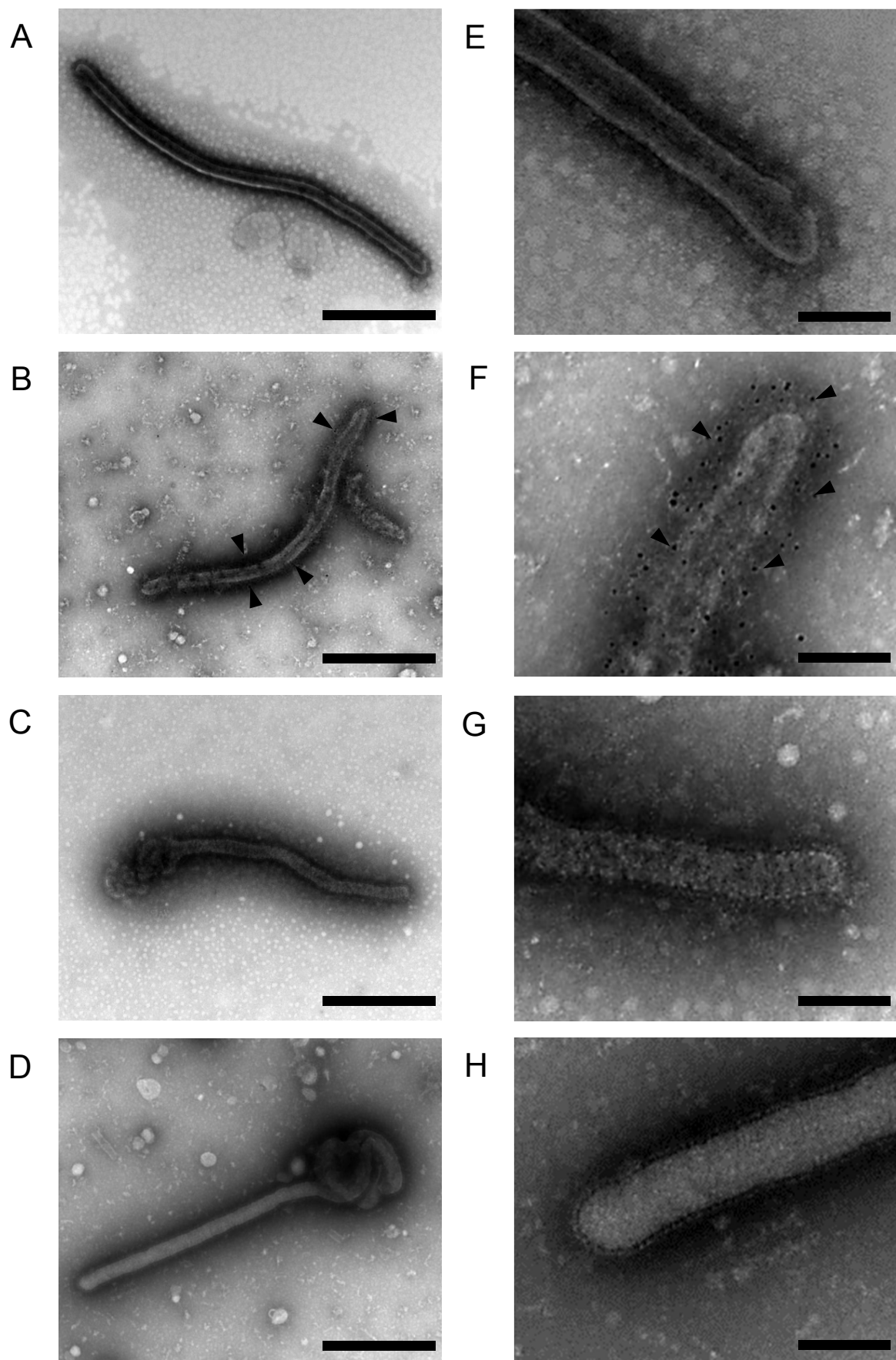
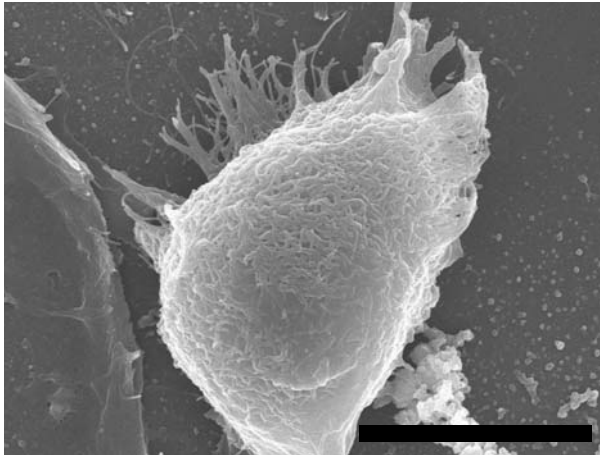
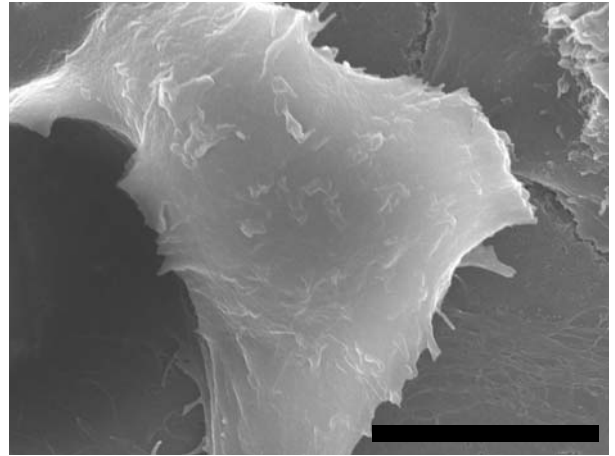


Fig. 2 Maruyama *et al.*

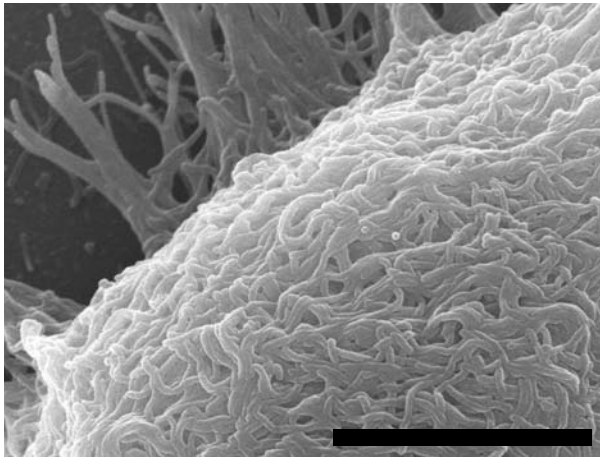
A



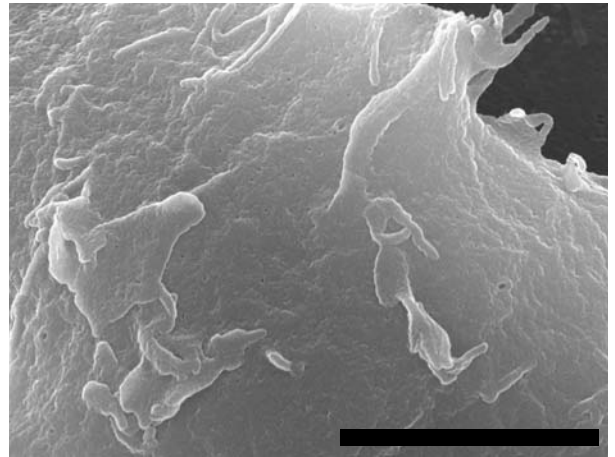
C



B



D



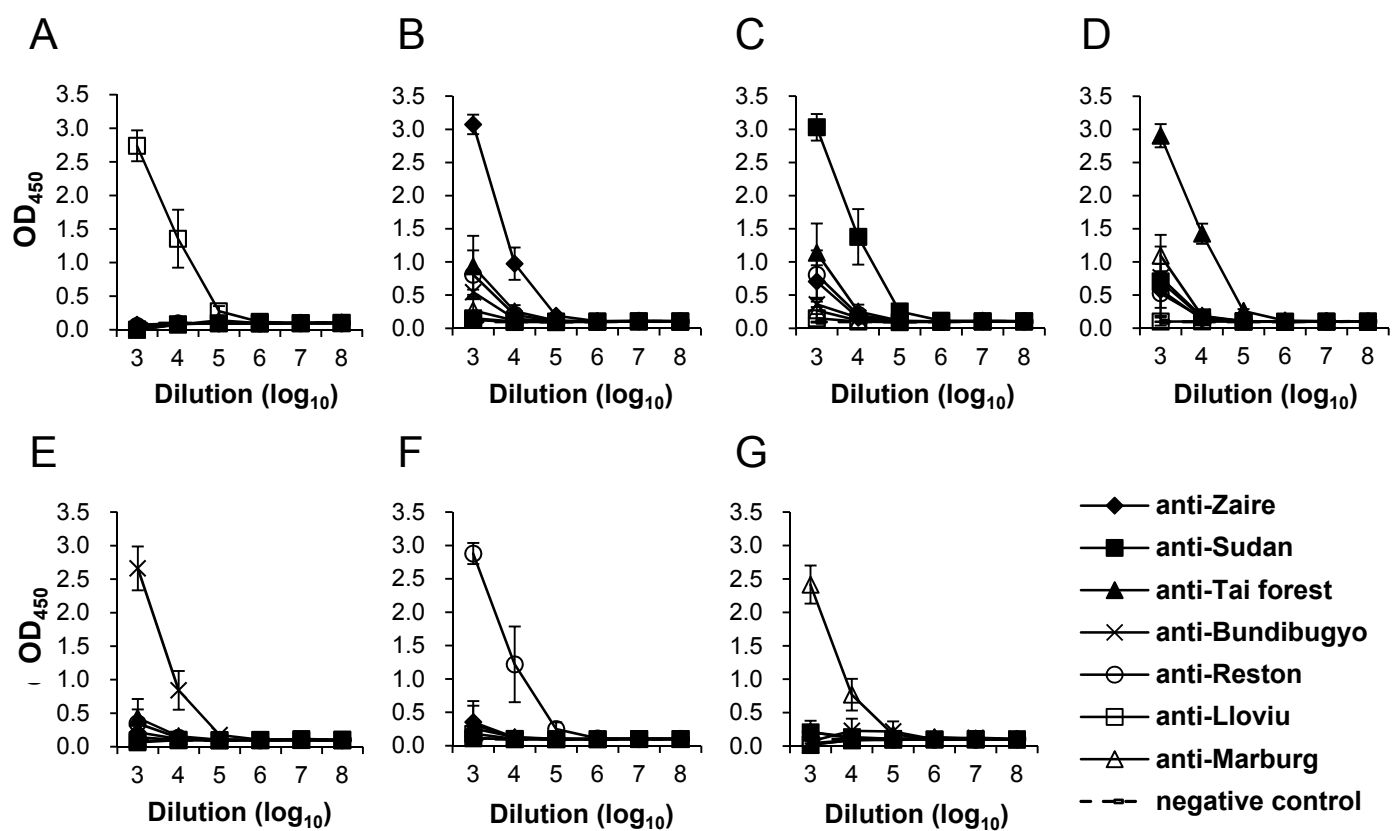


Fig. 4 Maruyama *et al.*

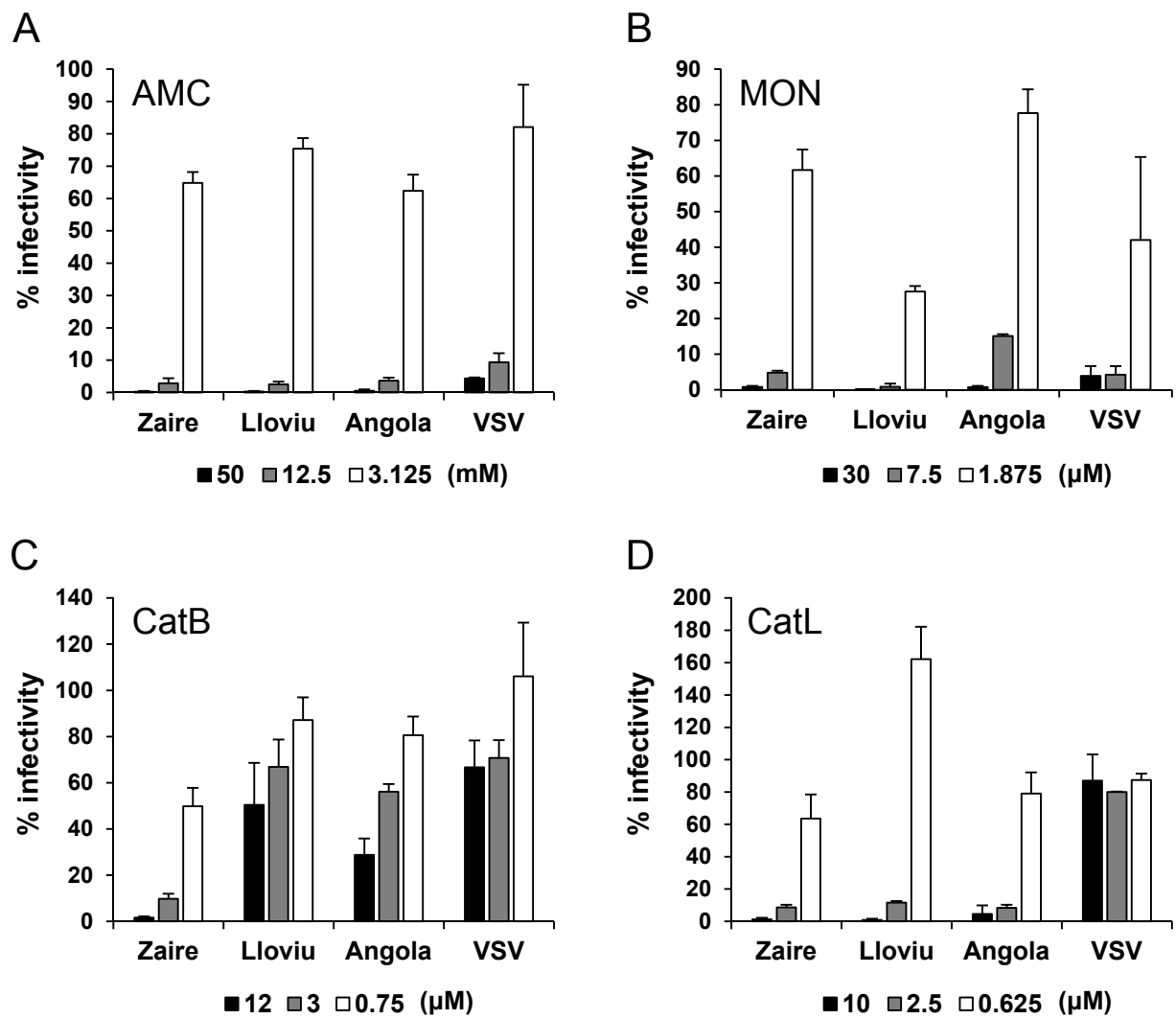


Fig. 5 Maruyama *et al.*

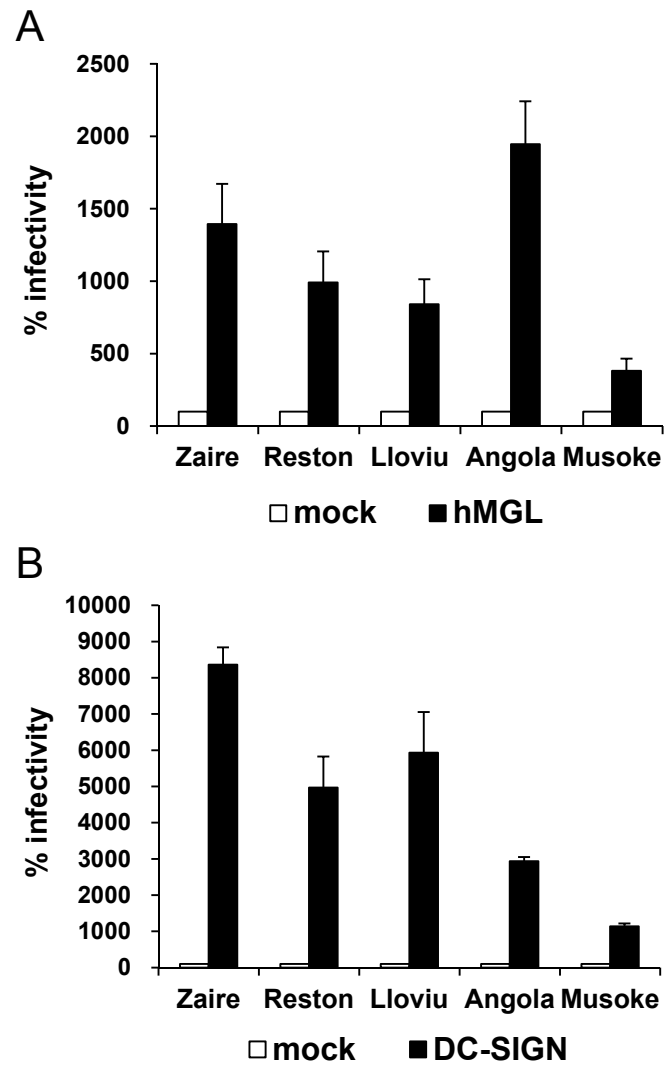


Fig. 6 Maruyama *et al.*

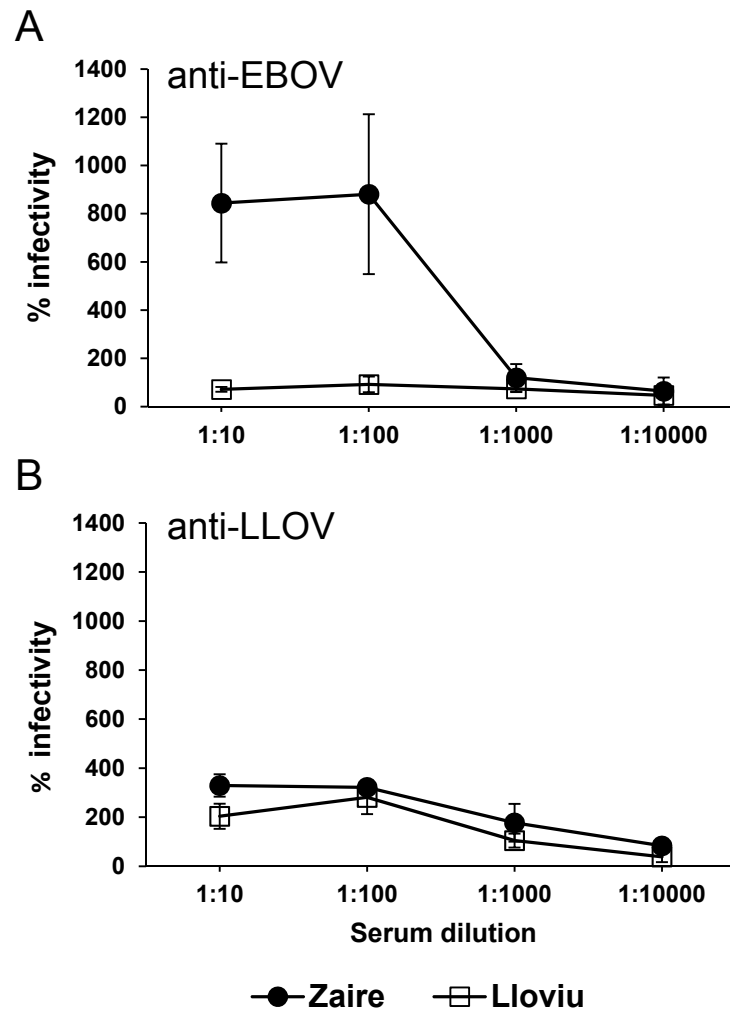


Fig. 7 Maruyama *et al.*

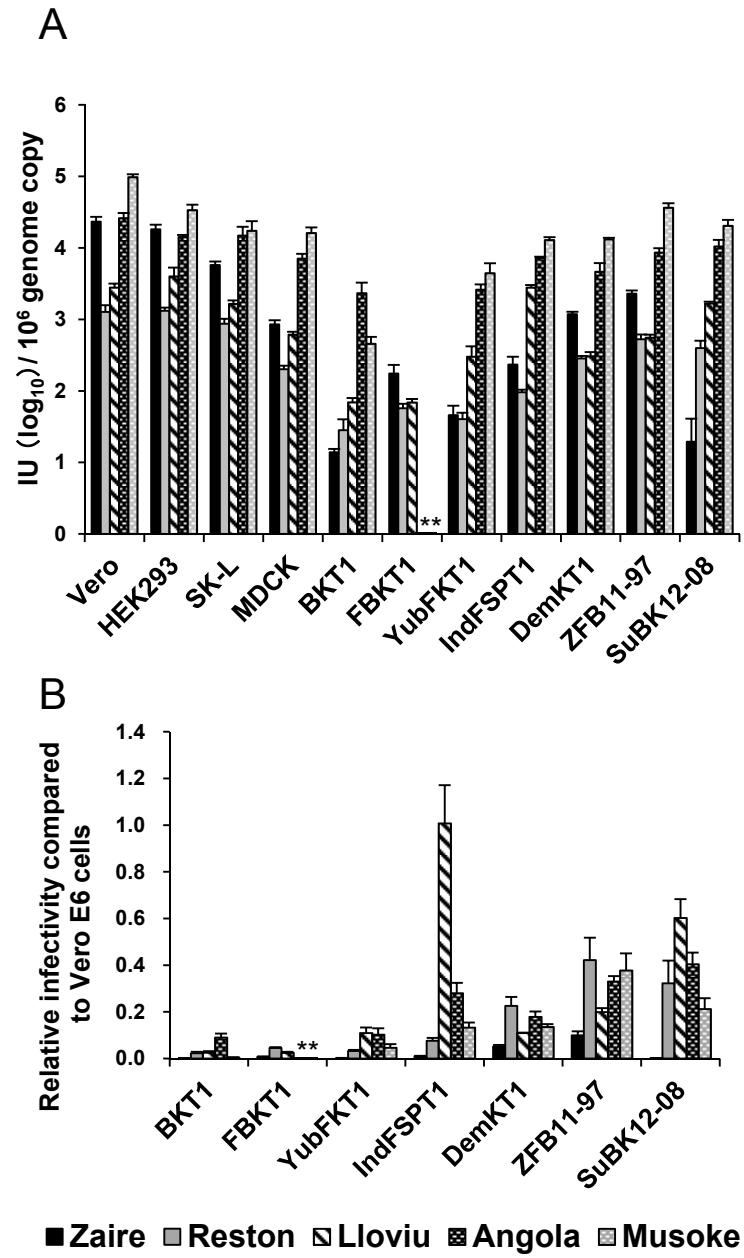


Fig. 8 Maruyama *et al.*