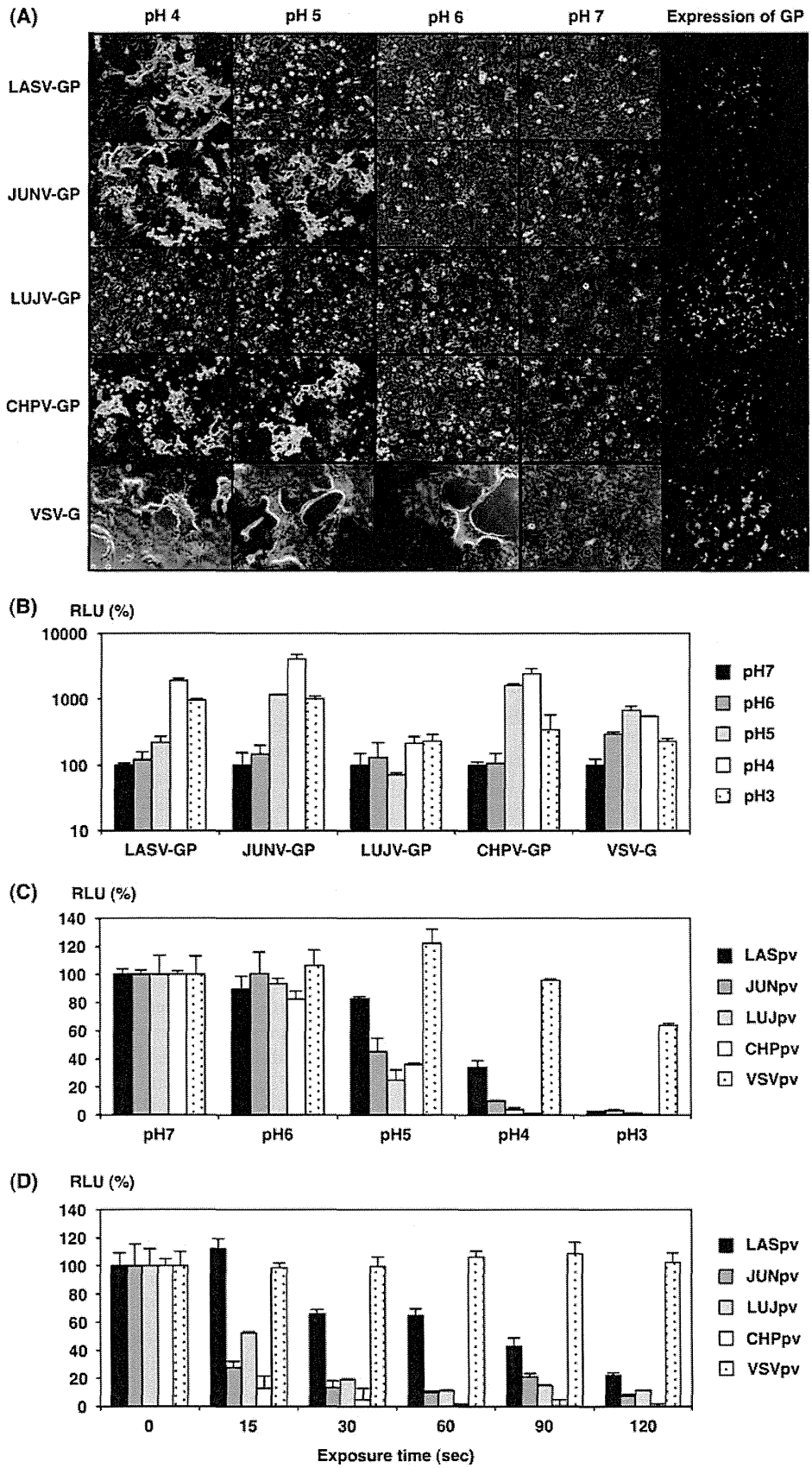

decreased in CHO/A101 cells and were recovered by the replenishment of NPC1 in CHO/A101-expressing NPC1 cells (Fig. 8D).

DISCUSSION

The development of safe alternative research tools for pathogenic arenaviruses that cause VHF in humans is very important for research on arenavirus diagnosis and for the development of prevention systems because classification of the viruses as BSL4 pathogens impedes their use in many countries. Toward this end, pseudotype virus systems based on VSV and retroviruses have been established. In the present study, we generated pseudotype VSVs bearing various arenaviral GPs, including the GP of a novel arenavirus (Lujo virus), and analyzed the involvement of arenaviral receptor candidates, fusion activities, and entry-mediated molecules.

Both VSV and retroviruses normally bud from the plasma membranes of the infected cells. The foreign viral envelope proteins expressed on the cell surface are therefore thought to be incorporated into the pseudotype particles during their budding. In the present study, although the presence of G1 could not be detected due to the absence of detectable antibodies, all of the carboxyl-terminally FOS-tagged arenaviral GPCs and G2s were detected in the cell lysates and purified virions, respectively (Fig. 1A). Arenaviral GPs may have been efficiently incorporated by VSV because of the presence of mature arenaviral GPs on the plasma membrane, which would make all of the pseudotype VSVs possessing arenaviral GPs highly infectious to the target cells. The fact that mature G2, but not GPC, was detected in the AREpv is consistent with a previous report that showed that only cleaved G1 and G2 were incorporated into the virions (38).

Previous studies have demonstrated the glycosylation of both LASV-GP and JUNV-GP by N-linked high-mannose-type oligosaccharide (39, 40). The glycosylation of VSV-G by complex-type oligosaccharide has also been demonstrated (26, 41). In the present study, all of the arenaviral GPs expressed in the cells and incorporated into the virions were sensitive to Endo H and PNGase F treatment (Fig. 1A), showing that they were glycosylated by high-mannose-type oligosaccharide. Though analysis of the glycosylation of live LUJV is needed, these data indicate that LUJV-GP was modified by the same glycosylation as the AREpv in the above-mentioned reports.

Previous studies have also demonstrated that several arenaviruses, including LASV, JUNV, and CHPV, and pseudotype viruses bearing their GPs were able to infect various types of cell lines. The AREpv were also able to infect almost all of the cell lines examined in the present study. However, LUJpv failed to infect NIH 3T3, NMuLi, or Molt-4 cells, while LASpv failed to infect Jurkat cells. It is known that Jurkat cells are not susceptible to LASV infection because they lack the function of O-mannosylation of αDG (11, 42). These results indicate that LASpv generated in this study represents the tropism of live LASV. Although comparison with live viruses is difficult, the entry mechanisms and characteristics of the envelope proteins of other pseudotype viruses are also believed to mimic those of live viruses. LUJV is known to be able to propagate in VeroE6 cells (43). The present study showed that, in addition to VeroE6 cells, both COS7 and Huh7 cells were highly susceptible to LUJpv infection. In contrast, no susceptibility was observed in mouse-derived cell lines (NIH 3T3 and NMuLi), but hamster-derived cell lines (BHK and CHO) were susceptible. The receptors and reservoirs of LUJV remain unclear. Although the characteristics of established cell lines may differ from those of the primary host cells, these results provide a hint for identifying the LUJV receptor and allow us to speculate with regard to its reservoir, since the expression of TfR1 from the reservoir rodent was shown to confer the highest susceptibility to many New World clade B pathogenic arenaviruses (44).

In the present study, we demonstrated that LUJpv entry was drastically decreased by the treatment of cells with chloroquine or chlorpromazine. These chemicals are widely known, not only as inhibitors of H^+-ATPase activation and clathrin-mediated endocytosis, but also as inducers of lipidosis. These phenomena make sense, because our data suggest that lipid metabolism plays an important role in LUJpv cell entry. Furthermore, given the use of chloroquine in the prevention and treatment of malaria in some areas of the world, it might be also available as a medicine to patients infected with LUJV.

Several studies have revealed αDG and TfR1 to be major cellular receptors for pathogenic Old World and New World clade B arenaviruses, respectively. In the present study, we demonstrated that LUJpv cell entry occurred independently of both αDG and TfR1. Although further experiments utilizing live LUJV to examine the entry and/or propagation mechanisms are needed, our results suggest the possibility that LUJV utilizes one or more novel receptors, but not αDG or TfR1.

Observation of syncytium formation and the cell-cell fusion assay provide simple, quantitative, and versatile tools to study viral-glycoprotein-mediated cell fusion (14, 45, 46). Low-pH-induced membrane fusion by arenaviral GPs has been the subject of previous studies (47, 48). In the present study, with the exception of LUJV-GP, cell fusion induced by arenaviral GPs initiated by treatment with low-pH buffer was clearly observed, both in syncytium formation and in reporter gene activities. As for LUJV-GP, no syncytium formation or reporter gene activities were observed

FIG 7 pH dependence on arenavirus GP for cell fusion and entry. (A) Syncytium formation of Huh7 cells transiently expressing arenaviral GPs or VSV-G after treatment with low-pH buffer. Huh7 cells were transfected with pCAG-LASV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-CHPV-GP-FOS, or pCAG-VSVG-FOS. At 24 h posttransfection, the cells were treated with citrate-phosphate buffer adjusted to the indicated pH value (pH 7, 6, 5, 4, or 3) for 2 min. Syncytium formation was determined by microscopic examination after 24 h (arenaviral GPs) or 8 h (VSV-G) incubation. Expression of arenaviral GPs or VSV-G at 24 h posttransfection was examined by immunofluorescence assay using anti-FLAG monoclonal antibody (right). (B) Quantitative cell fusion reporter assay. 293T cells transfected with pCAG-LASV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-CHPV-GP-FOS, or pCAG-VSVG-FOS, together with a plasmid encoding T7 RNA polymerase, were cocultured with Huh7 cells, which were transfected with a plasmid carrying a luciferase gene under the control of the T7 promoter. Cell fusion activity after exposure at the indicated pH for 2 min was determined by measuring RLU. (C) The relative infectivities of AREpv after exposure at the indicated pHs. LASpv, JUNpv, LUJpv, CHPpv, or VSVpv was exposed at the indicated pHs for 2 min. After neutralization with DMEM containing 10% FCS, the remaining infectivities of the pseudotypes for Huh7 cells were measured. The luciferase activities were determined at 24 h postinfection. (D) Effects of exposure time in low-pH buffer on AREpv infectivity. LASpv, JUNpv, LUJpv, CHPpv, or VSVpv was pretreated with pH 4 citrate-phosphate buffer for the indicated time (0, 15, 30, 60, 90 or 120 s). After neutralization, the pseudotypes were inoculated into Huh7 cells. The luciferase activities were determined at 24 h postinfection. The results shown are from three independent assays, with error bars representing standard deviations.

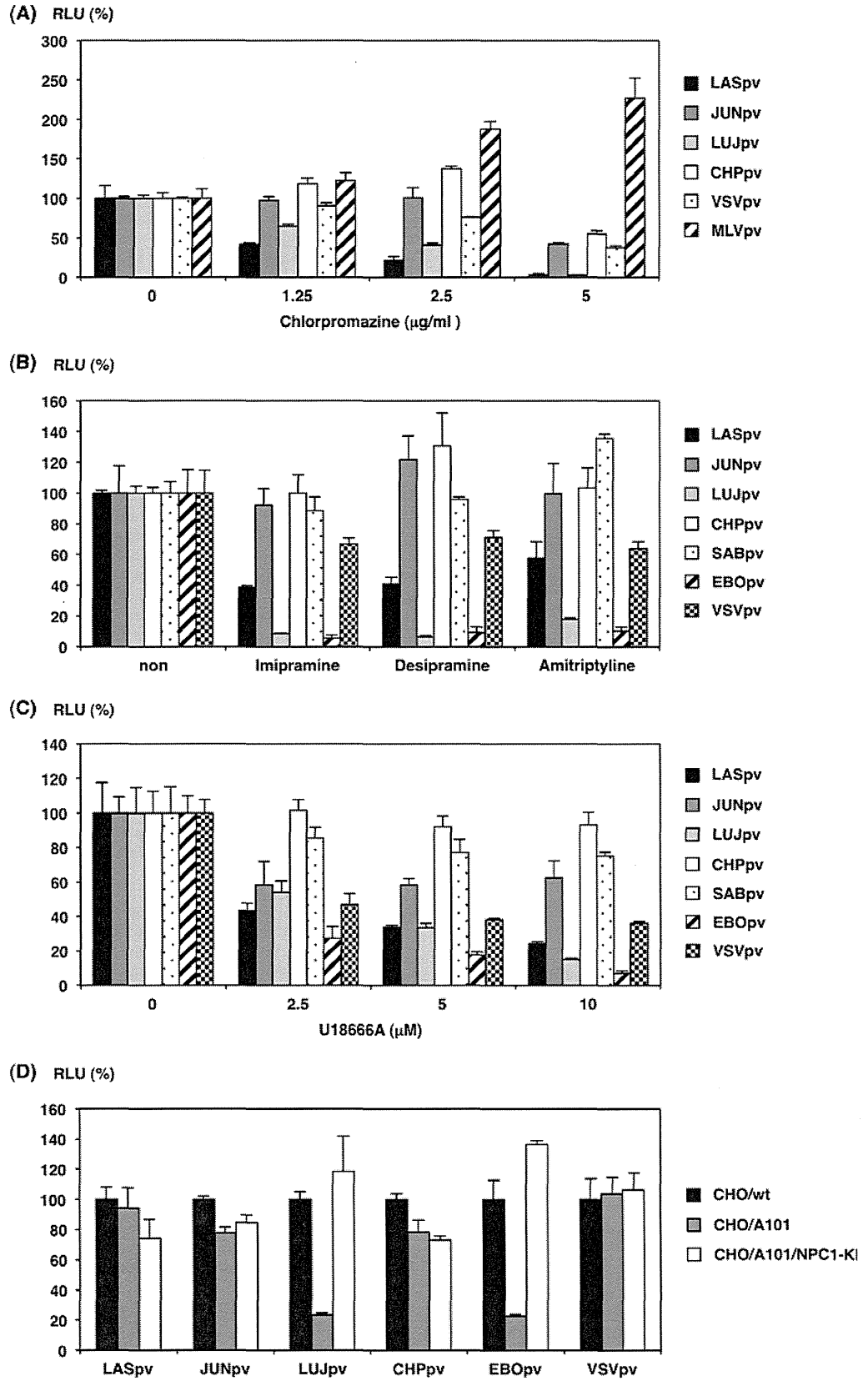

FIG 8 Involvement of cholesterol and sphingolipids in AREpv infection. (A to C) Infectivities of AREpv in Huh7 cells pretreated with the indicated concentrations of chlorpromazine (A); 10 μM imipramine, desipramine, or amitriptyline (B); or the indicated concentrations of U18666A (C). Huh7 cells were treated with each reagent and incubated at 37°C for 1 h. The cells were then inoculated with the pseudotype arenaviruses, EBOpv, MLVpv, or VSVpv, respectively. Their relative infectivities for the nontreated cells were determined at 24 h postinfection by measuring the luciferase activities. (D) Infectivities of pseudotype arenaviruses in wild-type CHO cells (CHO/wt), NPC1-deficient CHO cell mutants (CHO/A101), and CHO/A101 cells stably expressing FLAG-tagged NPC1 (CHO/A101/NPC1-KI). Their relative infectivities for the CHO/wt cells were obtained. The results shown are from three independent assays, with error bars representing standard deviations.

in the cells treated with low-pH buffer, despite the abundant expression of GP. Furthermore, immunofluorescence assays with and without permeabilized conditions revealed no difference between the cellular localization of GP in LUJV-GP and that of the other arenaviral GPs (data not shown). Moreover, LUJV-GP is considered to be present in the plasma membrane, because LUJV-GP was efficiently incorporated into virions and LUJpv, as well as other AREpv that exhibited high infectivity. In some of the enveloped viruses, such as Ebola virus or severe acute respiratory syndrome coronavirus (SARS-CoV), it is known that cleavage of the GP with the cysteine proteases cathepsins B and L is a prerequisite for membrane fusion (49–51). As far as we examined, the infectivity of LUJpv in Huh7 cells was not influenced by treatment with cathepsin B or L inhibitors (data not shown). Although we need to clarify whether extra cleavage or modification of LUJV-GP is necessary for membrane fusion, it is suggested that LUJV-GP shows unique characteristics among arenaviral GPs.

No visible syncytium formation was observed in the cells expressing Ebola virus GP under similar experimental conditions (data not shown). It was recently reported that in Ebola virus infection, the interaction of NPC1 with Ebola virus GP activated by the cathepsins is required for endosomal membrane fusion (31, 32). The results of the present study showed that LUJpv infection was inhibited, not only by lipidosis-inducing drugs, but also by the drug U18666A, which causes NPC1-like organelle defects. Furthermore, both LUJpv and EBOpv infections were abolished in NPC1-deficient cells, and these infectivities were recovered by the replenishment of NPC1 expression. Although it is currently not known whether LUJpv infection is directly involved in interaction with the NPC1 protein, the accumulation of cholesterol or some kinds of lipids may play important roles in the inhibition of LUJpv infection. Generally, NPC1 presents on cellular endosomes but not on the cell surface (52). If Lujo virus GP needs to interact with NPC1 in the same way as Ebola virus GP, this could be one of the reasons why cell-to-cell fusion did not occur in GP-expressing cells.

In conclusion, we generated replication-incompetent pseudotype arenaviruses possessing each arenaviral envelope glycoprotein, in particular, LUJpv, which is a novel surrogate model for the study of LUJV entry. In the present study, some of the analyses of LUJpv revealed that LUJV and its glycoprotein have unique characteristics for entry and fusion. Although LUJV is classified as a BSL4 pathogen, which makes it difficult to handle the authentic live virus, LUJpv and other pseudotype arenaviruses are quite useful for further detailed examination of arenaviral entry mechanisms.

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REFERENCES

1. **Bowen MD, Peters CJ, Nichol ST.** 1997. Phylogenetic analysis of the Arenaviridae: patterns of virus evolution and evidence for cospeciation between arenaviruses and their rodent hosts. Mol. Phylogenet. Evol. **8**:301–316. http://dx.doi.org/10.1006/mpev.1997.0436.
2. **Delgado S, Erickson BR, Agudo R, Blair PJ, Vallejo E, Albarino CG, Vargas J, Comer JA, Rollin PE, Ksiazek TG, Olson JG, Nichol ST.** 2008. Chapare virus, a newly discovered arenavirus isolated from a fatal hemorrhagic fever case in Bolivia. PLoS Pathog. **4**:e1000047. http://dx.doi.org/10.1371/journal.ppat.1000047.
3. **McCormick JB, Fisher-Hoch SP.** 2002. Lassa fever, p 75–109. *In* Oldstone MBA (ed), Arenaviruses I. Current topics in microbiology and immunology, vol 262. Springer, New York, NY. http://dx.doi.org/10.1007/978-3-642-56029-3_4.
4. **Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, Khristova ML, Weyer J, Swanepoel R, Egholm M, Nichol ST, Lipkin WI.** 2009. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from southern Africa. PLoS Pathog. **5**:e1000455. http://dx.doi.org/10.1371/journal.ppat.1000455.
5. **Burri DJ, da Palma JR, Kunz S, Pasquato A.** 2012. Envelope glycoprotein of arenaviruses. Viruses **4**:2162–2181. http://dx.doi.org/10.3390/v4102162.
6. **Cao W, Henry MD, Borrow P, Yamada H, Elder JH, Ravkov EV, Nichol ST, Compans RW, Campbell KP, Oldstone MB.** 1998. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. Science **282**:2079–2081. http://dx.doi.org/10.1126/science.282.5396.2079.
7. **Rojek JM, Spiropoulou CF, Campbell KP, Kunz S.** 2007. Old World and clade C New World arenaviruses mimic the molecular mechanism of receptor recognition used by alpha-dystroglycan's host-derived ligands. J. Virol. **81**:5685–5695. http://dx.doi.org/10.1128/JVI.02574-06.
8. **Flanagan ML, Oldenburg J, Reignier T, Holt N, Hamilton GA, Martin VK, Cannon PM.** 2008. New world clade B arenaviruses can use transferrin receptor 1 (TfR1)-dependent and -independent entry pathways, and glycoproteins from human pathogenic strains are associated with the use of TfR1. J. Virol. **82**:938–948. http://dx.doi.org/10.1128/JVI.01397-07.
9. **Radoshitzky SR, Abraham J, Spiropoulou CF, Kuhn JH, Nguyen D, Li W, Nagel J, Schmidt PJ, Nunberg JH, Andrews NC, Farzan M, Choe H.** 2007. Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. Nature **446**:92–96. http://dx.doi.org/10.1038/nature05539.
10. **Shimojima M, Kawaoka Y.** 2012. Cell surface molecules involved in infection mediated by lymphocytic choriomeningitis virus glycoprotein. J. Vet. Med. Sci. **74**:1363–1366. http://dx.doi.org/10.1292/jvms.12-0176.
11. **Shimojima M, Stroher U, Ebihara H, Feldmann H, Kawaoka Y.** 2012. Identification of cell surface molecules involved in dystroglycan-independent Lassa virus cell entry. J. Virol. **86**:2067–2078. http://dx.doi.org/10.1128/JVI.06451-11.
12. **Martinez MG, Bialecki MA, Belouzard S, Cordo SM, Candurra NA, Whittaker GR.** 2013. Utilization of human DC-SIGN and L-SIGN for entry and infection of host cells by the New World arenavirus, Junin virus. Biochem. Biophys. Res. Commun. **441**:612–617. http://dx.doi.org/10.1016/j.bbrc.2013.10.106.
13. **Goncalves AR, Moraz ML, Pasquato A, Helenius A, Lozach PY, Kunz S.** 2013. Role of DC-SIGN in Lassa virus entry into human dendritic cells. J. Virol. **87**:11504–11515. http://dx.doi.org/10.1128/JVI.01893-13.
14. **Takikawa S, Ishii K, Aizaki H, Suzuki T, Asakura H, Matsuura Y, Miyamura T.** 2000. Cell fusion activity of hepatitis C virus envelope proteins. J. Virol. **74**:5066–5074. http://dx.doi.org/10.1128/JVI.74.11.5066-5074.2000.
15. **Tani H, Morikawa S, Matsuura Y.** 2011. Development and applications of VSV vectors based on cell tropism. Front. Microbiol. **2**:272. http://dx.doi.org/10.3389/fmicb.2011.00272.
16. **Saijo M, Qing T, Niikura M, Maeda A, Ikegami T, Sakai K, Prehaud C, Kurane I, Morikawa S.** 2002. Immunofluorescence technique using HeLa cells expressing recombinant nucleoprotein for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J. Clin. Microbiol. **40**:372–375. http://dx.doi.org/10.1128/JCM.40.2.372-375.2002.
17. **Sayama Y, Demetria C, Saito M, Azul RR, Taniguchi S, Fukushi S, Yoshikawa T, Iizuka I, Mizutani T, Kurane I, Malbas FF, Jr, Lupisan S, Catbagan DP, Animas SB, Morales RG, Lopez EL, Dazo KR, Cruz MS, Olveda R, Saijo M, Oshitani H, Morikawa S.** 2012. A seroepidemiologic study of Reston ebolavirus in swine in the Philippines. BMC Vet. Res. **8**:82. http://dx.doi.org/10.1186/1746-6148-8-82.
18. **Higaki K, Ninomiya H, Sugimoto Y, Suzuki T, Taniguchi M, Niwa H, Pentchev PG, Vanier MT, Ohno K.** 2001. Isolation of NPC1-deficient Chinese hamster ovary cell mutants by gene trap mutagenesis. J. Biochem. **129**:875–880. http://dx.doi.org/10.1093/oxfordjournals.jbchem.a002932.
19. **Tani H, Komoda Y, Matsuo E, Suzuki K, Hamamoto I, Yamashita T,**

Moriishi K, Fujiyama K, Kanto T, Hayashi N, Owsianka A, Patel AH, Whitt MA, Matsuura Y. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. J. Virol. 81:8601–8612. http://dx.doi.org/10.1128/JVI.00608-07.

20. Taniguchi S, Sayama Y, Nagata N, Ikegami T, Miranda ME, Watanabe S, Iizuka I, Fukushi S, Mizutani T, Ishii Y, Saijo M, Akashi H, Yoshikawa Y, Kyuwa S, Morikawa S. 2012. Analysis of the humoral immune responses among cynomolgus macaque naturally infected with Reston virus during the 1996 outbreak in the Philippines. BMC Vet. Res. 8:189. http://dx.doi.org/10.1186/1746-6148-8-189.
21. Tani H, Shiokawa M, Kaname Y, Kambara H, Mori Y, Abe T, Moriishi K, Matsuura Y. 2010. Involvement of ceramide in the propagation of Japanese encephalitis virus. J. Virol. 84:2798–2807. http://dx.doi.org/10.1128/JVI.02499-09.
22. Fukushi S, Tani H, Yoshikawa T, Saijo M, Morikawa S. 2012. Serological assays based on recombinant viral proteins for the diagnosis of arenavirus hemorrhagic fevers. Viruses 4:2097–2114. http://dx.doi.org/10.3390/v4102097.
23. Ward JH, Kushner JP, Kaplan J. 1982. Transferrin receptors of human fibroblasts. Analysis of receptor properties and regulation. Biochem. J. 208:19–26.
24. Iha K. 2013. Pseudotyped vesicular stomatitis virus for analysis of entry of arenaviruses and its application to serodiagnosis of Argentine hemorrhagic fever. .Ph.D. thesis. University of Tokyo, Tokyo, Japan.
25. Spiro MJ, Spiro RG. 2000. Use of recombinant endomannosidase for evaluation of the processing of N-linked oligosaccharides of glycoproteins and their oligosaccharide-lipid precursors. Glycobiology 10:521–529. http://dx.doi.org/10.1093/glycob/10.5.521.
26. Spiro MJ, Spiro RG. 2001. Release of polymannose oligosaccharides from vesicular stomatitis virus G protein during endoplasmic reticulum-associated degradation. Glycobiology 11:803–811. http://dx.doi.org/10.1093/glycob/11.10.803.
27. Glushakova SE, Lukashevich IS. 1989. Early events in arenavirus replication are sensitive to lysosomotropic compounds. Arch. Virol. 104:157–161. http://dx.doi.org/10.1007/BF01313817.
28. Castilla V, Palermo LM, Coto CE. 2001. Involvement of vacuolar proton ATPase in Junin virus multiplication. Arch. Virol. 146:251–263. http://dx.doi.org/10.1007/s007050170173.
29. Cosset FL, Marianneau P, Verney G, Gallais F, Tordo N, Pecheur EI, ter Meulen J, Deubel V, Bartosch B. 2009. Characterization of Lassa virus cell entry and neutralization with Lassa virus pseudoparticles. J. Virol. 83:3228–3237. http://dx.doi.org/10.1128/JVI.01711-08.
30. Kunz S, Rojek JM, Kanagawa M, Spiropoulou CF, Barresi R, Campbell KP, Oldstone MB. 2005. Posttranslational modification of alpha-dystroglycan, the cellular receptor for arenaviruses, by the glycosyltransferase LARGE is critical for virus binding. J. Virol. 79:14282–14296. http://dx.doi.org/10.1128/JVI.79.22.14282-14296.2005.
31. Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, Kuehne AI, Kranzusch PJ, Griffin AM, Ruthel G, Dal Cin P, Dye JM, Whelan SP, Chandran K, Brummelkamp TR. 2011. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. Nature 477:340–343. http://dx.doi.org/10.1038/nature10348.
32. Miller EH, Obernosterer G, Raaben M, Herbert AS, Deffieu MS, Krishnan A, Ndungo E, Sandesara RG, Carette JE, Kuehne AI, Ruthel G, Pfeffer SR, Dye JM, Whelan SP, Brummelkamp TR, Chandran K. 2012. Ebola virus entry requires the host-programmed recognition of an intracellular receptor. EMBO J. 31:1947–1960. http://dx.doi.org/10.1038/emboj.2012.53.
33. Miller ME, Adhikary S, Kolokoltsov AA, Davey RA. 2012. Ebolavirus requires acid sphingomyelinase activity and plasma membrane sphingomyelin for infection. J. Virol. 86:7473–7483. http://dx.doi.org/10.1128/JVI.00136-12.
34. Lange Y, Ye J, Steck TL. 2012. Activation mobilizes the cholesterol in the late endosomes-lysosomes of Niemann Pick type C cells. PLoS One 7:e30051. http://dx.doi.org/10.1371/journal.pone.0030051.
35. Martinez MG, Cordo SM, Candurra NA. 2007. Characterization of Junin arenavirus cell entry. J. Gen. Virol. 88:1776–1784. http://dx.doi.org/10.1099/vir.0.82808-0.
36. Liscum L, Faust JR. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one. J. Biol. Chem. 264:11796–11806.
37. Cenedella RJ. 2009. Cholesterol synthesis inhibitor U18666A and the role of sterol metabolism and trafficking in numerous pathophysiological processes. Lipids 44:477–487. http://dx.doi.org/10.1007/s11745-009-3305-7.
38. Lenz O, ter Meulen J, Klenk HD, Seidah NG, Garten W. 2001. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. Proc. Natl. Acad. Sci. U. S. A. 98:12701–12705. http://dx.doi.org/10.1073/pnas.221447598.
39. Albarino CG, Bergeron E, Erickson BR, Khristova ML, Rollin PE, Nichol ST. 2009. Efficient reverse genetics generation of infectious Junin viruses differing in glycoprotein processing. J. Virol. 83:5606–5614. http://dx.doi.org/10.1128/JVI.00276-09.
40. Eichler R, Lenz O, Garten W, Strecker T. 2006. The role of single N-glycans in proteolytic processing and cell surface transport of the Lassa virus glycoprotein GP-C. Virol. J. 3:41. http://dx.doi.org/10.1186/1743-422X-3-41.
41. Marozin S, Altomonte J, Apfel S, Dinh PX, De Toni EN, Rizzani A, Nussler A, Kato N, Schmid RM, Pattnaik AK, Ebert O. 2012. Posttranslational modification of vesicular stomatitis virus glycoprotein, but not JNK inhibition, is the antiviral mechanism of SP600125. J. Virol. 86:4844–4855. http://dx.doi.org/10.1128/JVI.06649-11.
42. Rojek JM, Campbell KP, Oldstone MB, Kunz S. 2007. Old World arenavirus infection interferes with the expression of functional alpha-dystroglycan in the host cell. Mol. Biol. Cell 18:4493–4507. http://dx.doi.org/10.1091/mbc.E07-04-0374.
43. Bergeron E, Chakrabarti AK, Bird BH, Dodd KA, McMullan LK, Spiropoulou CF, Nichol ST, Albarino CG. 2012. Reverse genetics recovery of Lujo virus and role of virus RNA secondary structures in efficient virus growth. J. Virol. 86:10759–10765. http://dx.doi.org/10.1128/JVI.01144-12.
44. Abraham J, Kwong JA, Albarino CG, Lu JG, Radoshitzky SR, Salazar-Bravo J, Farzan M, Spiropoulou CF, Choe H. 2009. Host-species transferrin receptor 1 orthologs are cellular receptors for nonpathogenic new world clade B arenaviruses. PLoS Pathog. 5:e1000358. http://dx.doi.org/10.1371/journal.ppat.1000358.
45. Suenaga T, Satoh T, Somboonthum P, Kawaguchi Y, Mori Y, Arase H. 2010. Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses. Proc. Natl. Acad. Sci. U. S. A. 107:866–871. http://dx.doi.org/10.1073/pnas.0913351107.
46. Tanaka Y, Suenaga T, Matsumoto M, Seya T, Arase H. 2013. Herpesvirus 6 glycoproteins B (gB), gH, gL, and gQ are necessary and sufficient for cell-to-cell fusion. J. Virol. 87:10900–10903. http://dx.doi.org/10.1128/JVI.01427-13.
47. Castilla V, Mersich SE. 1996. Low-pH-induced fusion of Vero cells infected with Junin virus. Arch. Virol. 141:1307–1317. http://dx.doi.org/10.1007/BF01718832.
48. Klewitz C, Klenk HD, ter Meulen J. 2007. Amino acids from both N-terminal hydrophobic regions of the Lassa virus envelope glycoprotein GP-2 are critical for pH-dependent membrane fusion and infectivity. J. Gen. Virol. 88:2320–2328. http://dx.doi.org/10.1099/vir.0.82950-0.
49. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P. 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. U. S. A. 102:11876–11881. http://dx.doi.org/10.1073/pnas.0505577102.
50. 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. http://dx.doi.org/10.1126/science.1110656.
51. Schornberg K, Matsuyama S, Kabsch K, Delos S, Bouton A, White J. 2006. Role of endosomal cathepsins in entry mediated by the Ebola virus glycoprotein. J. Virol. 80:4174–4178. http://dx.doi.org/10.1128/JVI.80.8.4174-4178.2006.
52. Garver WS, Heidenreich RA, Erickson RP, Thomas MA, Wilson JM. 2000. Localization of the murine Niemann-Pick C1 protein to two distinct intracellular compartments. J. Lipid Res. 41:673–687.

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Safety of Attenuated Smallpox Vaccine LC16m8 in Immunodeficient Mice

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Freeze-dried live attenuated smallpox vaccine LC16m8 prepared in cell culture has been the sole smallpox vaccine licensed in Japan since 1975 and was recently recommended as a WHO stockpile vaccine. We evaluated the safety of recently remanufactured lots of LC16m8 using a series of immunodeficient mouse models. These models included suckling mice, severe combined immunodeficiency disease (SCID) mice, and wild-type mice treated with cyclosporine. LC16m8 showed extremely low virulence in each of the three mouse models compared with that of its parental strains, Lister and LC16mO. These results provide further evidence that LC16m8 is one of the safest replication-competent smallpox vaccines in the world and may be considered for use in immunodeficient patients.

Smallpox was eradicated using vaccinia virus vaccines through the smallpox eradication program led by the World Health Organization (WHO); the WHO certified the eradication of smallpox in 1980 (1, 2). Vaccinia virus strains used in this program were mostly derived from the Lister and New York City Board of Health (NYCBH) strains. Data gathered in 1968 before eradication showed that the rate of serious adverse events, such as postvaccinial encephalitis, was about 20 per 1 million primary subjects (3). Therefore, the WHO recommended that vaccinations be stopped after eradication.

The risk of bioterrorism using smallpox or other lethal agents is a major concern that has increased since the 11 September 2001 incident in New York, New York, USA. Among the pathogens that might be used in bioterrorism, variola virus is one of the most feared. Therefore, a safer smallpox vaccine is an important goal.

Serious attempts have been made to develop lower-virulent replication-competent vaccines, such as ACAM2000, derived from NYCBH Dryvax (Wyeth Pharmaceuticals) (4), and modified vaccinia Ankara (MVA) (5, 6). A nonreplicating vaccine has also been developed from the NYCBH strain. However, ACAM2000 was reported to induce myopericarditis (7, 8) and probably has virulence similar to that of other NYCBH vaccines. The replication-incompetent vaccines may have relatively poor immunogenicity (5, 6), and replication-competent MVA requires repeated vaccination for optimal immunity and maintains high levels of serum antibody for a relatively short period.

In the late 1970s, Hashizume and coworkers developed one of the safest replication-competent vaccines, LC16m8, from the original Lister strain (9, 10). This vaccine is a freeze-dried vaccine prepared in cell culture and is the sole smallpox vaccine licensed in Japan. In the original study of Hashizume et al., LC16m8 was selected as a temperature-sensitive small plaque- and small pock-forming clone (9, 10). A rabbit skin proliferation study and a neurovirulence study in which LC16m8 and Lister viruses were inoculated into the thalamus of cynomolgus monkeys showed very low pathogenicity of LC16m8 compared with that of Lister. A clinical evaluation of 90,000 infants immunized during the initial development of LC16m8 from 1973 to early 1976 showed no encephalitis or other serious adverse events after vaccination. No major differences exist in the immunogenicity of LC16m8 compared with that of conventional first-generation smallpox vaccines (3).

The LC16m8 vaccine has now been manufactured and maintained as a stockpile in Japan. A 2013 WHO Strategic Advisory Group of Experts (SAGE) meeting on immunization recommended both licensed ACAM2000 (second-generation vaccine) and LC16m8 (third-generation vaccine) as the preferred WHO stockpile vaccines (11). Based on this WHO recommendation, use of LC16m8 as a WHO stockpile vaccine for many subjects, including immunodeficient subjects in the future, is possible.

A wide epidemic of monkeypox was reported in areas of western and central Africa where a high prevalence of HIV infection exists (12). In this case, whether LC16m8 is applicable to the population, including immunodeficient subjects such as those infected with HIV, has become an important question to be answered. We report here the safety of this vaccine, focusing on experiments done with three immunodeficient mouse models inoculated with newly manufactured LC16m8 to address this question.

MATERIALS AND METHODS

Vaccine and virus strains. We used Kaketsuken-manufactured vaccine (LC16-Kaketsuken, LC16m8) prepared in a culture of rabbit kidney cells and freeze-dried. We obtained Lister (Elstree) and LC16mO, the parental strains of LC16m8, from the Chiba Serum Institute (Chiba, Japan) and propagated them in VeroE6 (Vero C1008) cells (ATCC CRL-156) and RK-13 cells, respectively. All three of these vaccine strains were titrated in VeroE6 cells and were used to vaccinate control groups of mice in the experiments.

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TABLE 1 Mouse groups used for CsA experiments

Group	Vaccine	CsA[a] dose (mg/kg/day)
1	Nothing	0
2	Vaccine diluent	25
3	Vaccine diluent	50
4	LC16m8	0
5	LC16m8	25
6	LC16m8	50
7	Lister	0
8	Lister	25
9	Lister	50

[a] CsA, cyclosporine.

TABLE 2 Criteria for scoring clinical observations of mice[a]

Score	Clinical symptom criteria
0	No clinical symptoms
1	Rough coat, rash, nervous behavior
2	Spiky hair, pock formation (no suppuration)
3	Pock formation (suppuration), loss of hair, hunched over, inactive, slow moving
4	Paralysis, moribund, abnormal behavior (stumbling, etc.)
5	Dead

[a] The evaluation of clinical symptoms was expressed as a score for each mouse. Each group's total clinical score for every observation day was expressed as the sum of each mouse score.

Animals. We purchased a total of 120 ICR suckling mice (3 to 5 days old) from Japan SLC (Shizuoka, Japan). A total of 40 severe combined immunodeficiency disease (SCID) mice (CD-17/1cr-SCID, 6-week-old females) and a total of 54 wild-type C57BL/6 mice (7-week-old females) were obtained from Charles River Japan (Kanagawa, Japan). All mouse studies were approved by the Kaketsuken Institutional Animal Care and Use Committee.

Immunosuppressive agent. We obtained cyclosporine (CsA) from Wako Pure Chemical Industries (Osaka, Japan). We prepared dosing solutions daily by diluting the CsA solution with a water solution containing 5% ethanol and 1% Tween 80 for the final injection.

Neurovirulence test with suckling mice. Ten suckling mice in each of three groups were inoculated intracerebrally with $10^{3.3}$ PFU of either LC16m8, LC16mO, or Lister. These mice were observed every day for 21 days after inoculation. We calculated the survival ratio and mean survival time (days) by observing the mice for 21 days after inoculation. Statistical analyses were done using Fisher's exact method and the log rank method. The neurovirulence test was done according to the WHO recommendations (13).

LD_{50} test with suckling mice. Groups of 10 suckling mice were each inoculated intracerebrally with graded doses from $10^{3.3}$ to $10^{5.3}$ PFU of LC16m8 and from $10^{1.3}$ to $10^{3.3}$ PFU of LC16mO and of Lister. These mice were observed every day for 21 days after inoculation. The 50% lethal dose (LD_{50}) was calculated by the probit method in mice that died up to 21 days after inoculation.

Test with SCID mice. Ten mice in each of the three SCID mouse groups were inoculated intraperitoneally with $10^{5.3}$ PFU of either LC16m8, LC16mO, or Lister. Ten additional mice were inoculated intraperitoneally with a saline as a control. These mice were observed for 120 days after inoculation.

Test with CsA-treated mice. Mice were divided into a total of 9 groups with 6 mice each as shown in Table 1. Mice were injected subcutaneously every day for 15 days (from day 1 before inoculation to day 14 after inoculation) with 25 or 50 mg/kg of body weight/day CsA or a water solution containing 5% ethanol and 1% Tween 80 as a control. The mice in groups 1, 2, and 3 did not receive vaccine inoculation throughout the experimental period, and the changes in immunocyte numbers after treatment with CsA were examined on day 14 after CsA injection. The mice in groups 4, 5, 6, 7, 8, and 9 were inoculated intraperitoneally with 10^7 PFU of Lister or LC16m8 (total of six groups). The clinical symptoms of the mice in all 9 groups were recorded every day during the 14-day experimental period. Table 2 shows the scoring system used in this study, which was designed by us based on the scoring system used for general acute toxicity tests in wild-type mice but with minor modifications.

The change in immunocyte numbers for the three control groups with no CsA treatment or CsA treatment, but not receiving any vaccine, on day 14 after inoculation was evaluated by staining splenocytes with (i) anti-$CD4^+$ T cell antibodies conjugated with phycoerythrin (PE) (anti-CD4-PE), (ii) anti-CD3 antibodies conjugated with fluorescein isothiocyanate (FITC) (anti-CD3-FITC), (iii) anti-$CD8^+$ T cell antibodies conjugated with PE (anti-CD8-PE) together with anti-CD3-FITC antibodies, and (iv) anti-B cell antibodies [anti-CD45R(B220)-PE]. Only spleen was studied because CsA is reported to affect the spleen and lymph nodes in a similar way (14). These four antibodies were purchased from Becton Dickinson, NJ. Stained splenocytes were analyzed by using a fluorescence-activated cell sorter (FACScan, Becton, Dickinson).

RESULTS AND DISCUSSION

We prepared new stockpile LC16m8 lots and used them in a series of safety tests that were essentially the same as the tests done by Hashizume and coworkers (9, 10) with rabbits and monkeys. Our results were also essentially the same as the results of Hashizume et al. (data not shown).

Survival study in suckling mice. After we inoculated suckling mice intracerebrally with $10^{3.3}$ PFU of LC16m8, LC16mO, or Lister, the survival ratios during the 21 days of observation were 7:10 (70%) for LC16m8, 1:10 (10%) for LC16mO, and 1:10 (10%) for Lister (Fig. 1). The difference in the survival ratios between LC16m8 and either of the two unattenuated strains was significant ($P = 0.02$, Fisher's exact test). The mean survival times were 17.1 days for LC16m8 versus only 6.1 days for LC16mO and 6.3 days for Lister ($P = 0.001$, log rank test). The LD_{50}s calculated at 21 days after inoculation were $10^{5.1}$ PFU for LC16m8 versus $10^{1.6}$ PFU for LC16mO and $10^{1.4}$ PFU for Lister. Thus, the LD_{50} for LC16m8 was >1,000-fold higher than that for the unattenuated strains (Table 3).

Survival and clinical symptoms of SCID mice. The three vaccine strains were inoculated intraperitoneally into SCID mice. Figure 2A shows the survival curve. The survival ratios were 0:10 (0%) for Lister and for LC16mO and 10:10 (100%) for LC16m8. The increase in mean survival times between LC16m8 (>120 days) and Lister (30.8 days) was statistically significant ($P < 0.001$, log rank test); the difference between LC16m8 (>120 days) and LC16mO (24.5 days) was also statistically significant ($P < 0.001$, log rank test).

Figure 2B shows the group's average percentage of weight gain or loss patterns from each animal's weight on the inoculation day. Lister- and LC16mO-inoculated mice showed a marked decrease in body weight from day 12 after inoculation. All mice were euthanized by CO_2 gas when the weight loss reached ≥25%. No significant weight loss was observed in LC16m8-inoculated mice nor in placebo-inoculated mice.

Figure 2C shows the clinical symptom data for these mice. Lister- and LC16mO-inoculated mice began to show clinical symptoms around day 10 after inoculation followed by an abrupt increase in severity, reaching maximal levels between 20 and 30 days after inoculation, whereas the LC16m8-inoculated mice showed no recognizable clinical symptoms.

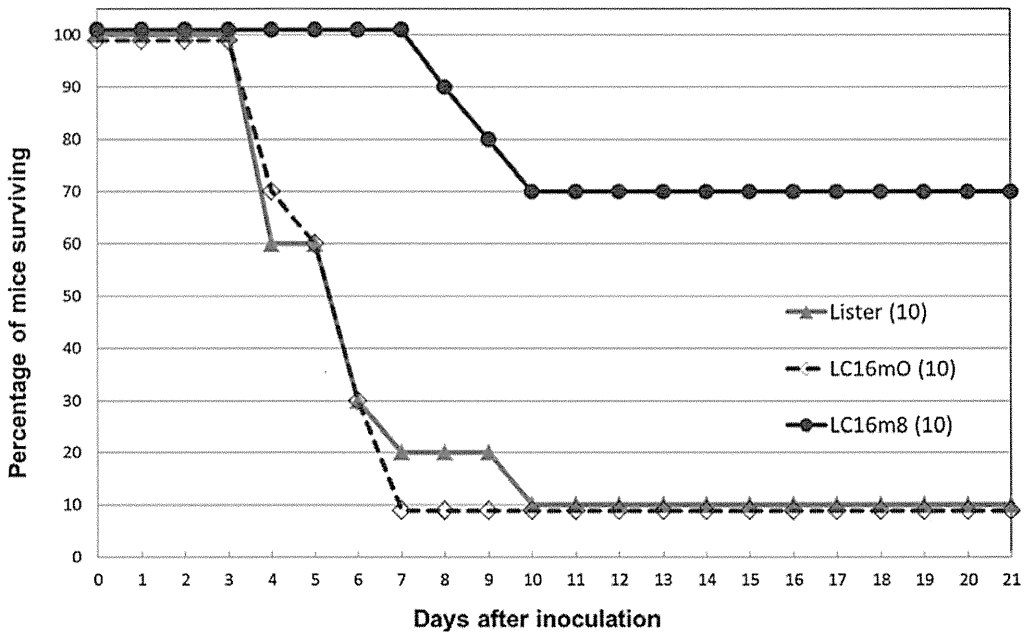

FIG 1 Effects of vaccination with Lister, LC16mO, or LC16m8 vaccine on ICR suckling mice. Each vaccine was inoculated intracerebrally (see details in the text). The numbers in parentheses shows the number of animals in each experimental group.

Clinical symptoms of CsA-treated mice. Lister and LC16m8 were inoculated intraperitoneally into mice in which the immune system was suppressed by CsA. For the mice not receiving any vaccine, the effects of CsA on the immunocyte numbers were evaluated: the numbers of $CD4^+$ T cells and $CD8^+$ T cells decreased by about 20% compared with those in the non-CsA-treated mice, but little change was observed in the numbers of B cells (Fig. 3A). No mice died during the observation period. Lister-inoculated mice treated with CsA developed severe vaccinia-related symptoms, including pock formation, rash, or both. LC16m8-inoculated mice in either CsA-treated or nontreated groups developed no clinical symptoms (Fig. 3B).

These studies indicate that the LC16m8 virus has highly attenuated properties in immunodeficient mice. This attenuation is mainly caused by a mutation in the *B5R* gene coding for an extracellular envelope protein necessary for rapid propagation of the virus (15–17). Although LC16m8 lacks the normal *B5R* gene product (B5 protein), it has a strong protective ability against infection by virulent viruses in various immunodeficient mouse models (18–20). In this regard, we previously studied (18) the effect of Dryvax or LC16m8 by administration by skin scarification to macaques depleted systemically of T or B cells. B cell depletion did not affect the size of the skin lesions induced by either vaccine. However, while depletion of both $CD4^+$ and $CD8^+$ T cells had no adverse effects on LC16m8-vaccinated animals, it caused progressive vaccinia in macaques immunized with Dryvax. As both Dryvax and LC16m8 vaccines protect healthy macaques from a lethal monkeypox intravenous challenge, our data identified LC16m8 as a safer and effective alternative to ACAM2000 and Dryvax vaccines for immunocompromised individuals.

TABLE 3 Neurovirulence LD_{50} of vaccinia viruses in suckling mice inoculated by the intracerebral route

Strain	Inoculation dose (log PFU/mouse)	Mortality (no. of deaths/total no.)	LD_{50}[a] (log PFU/mouse)
Lister	3.3	10/10	1.4
	2.3	7/10	
	1.3	5/10	
LC16mO	3.3	10/10	1.6
	2.3	9/10	
	1.3	3/10	
LC16m8	5.3	6/10	5.1
	4.3	2/10	
	3.3	0/10	

[a] LD_{50}, 50% lethal dose.

We have found no report on pathogenesis directly comparing LC16m8 and MVA. However, a smallpox vaccine strain (m8Δ) constructed by modifying LC16m8 showed lower pathogenicity similar to that of MVA in SCID mice (19). This modification only augmented the genetic stability by lowering the risk of the occurrence of a *B5R* gene revertant without affecting its pathogenicity, suggesting that LC16m8 and MVA have comparable pathogenicity. As noted above, MVA has poor immunogenicity (20).

Based on the above-mentioned facts, we consider LC16m8 to be one of the best candidate vaccines usable in the case of attack by bioterrorists, and the SAGE recommended both ACAM2000 (extended-spectrum vaccine) and LC16m8 (broad-spectrum vaccine) as preferred WHO stockpile vaccines (11).

This study on the safety of LC16m8 by use of suckling mice and immunocompromised mice not only confirms the safety of the LC16m8 vaccine but also supports the idea that LC16m8 could be usable for immunodeficient subjects, including those infected with HIV, which is important in relation to the recent epidemics of monkeypox in western and central Africa.

Infection with monkeypox has increased greatly in areas of

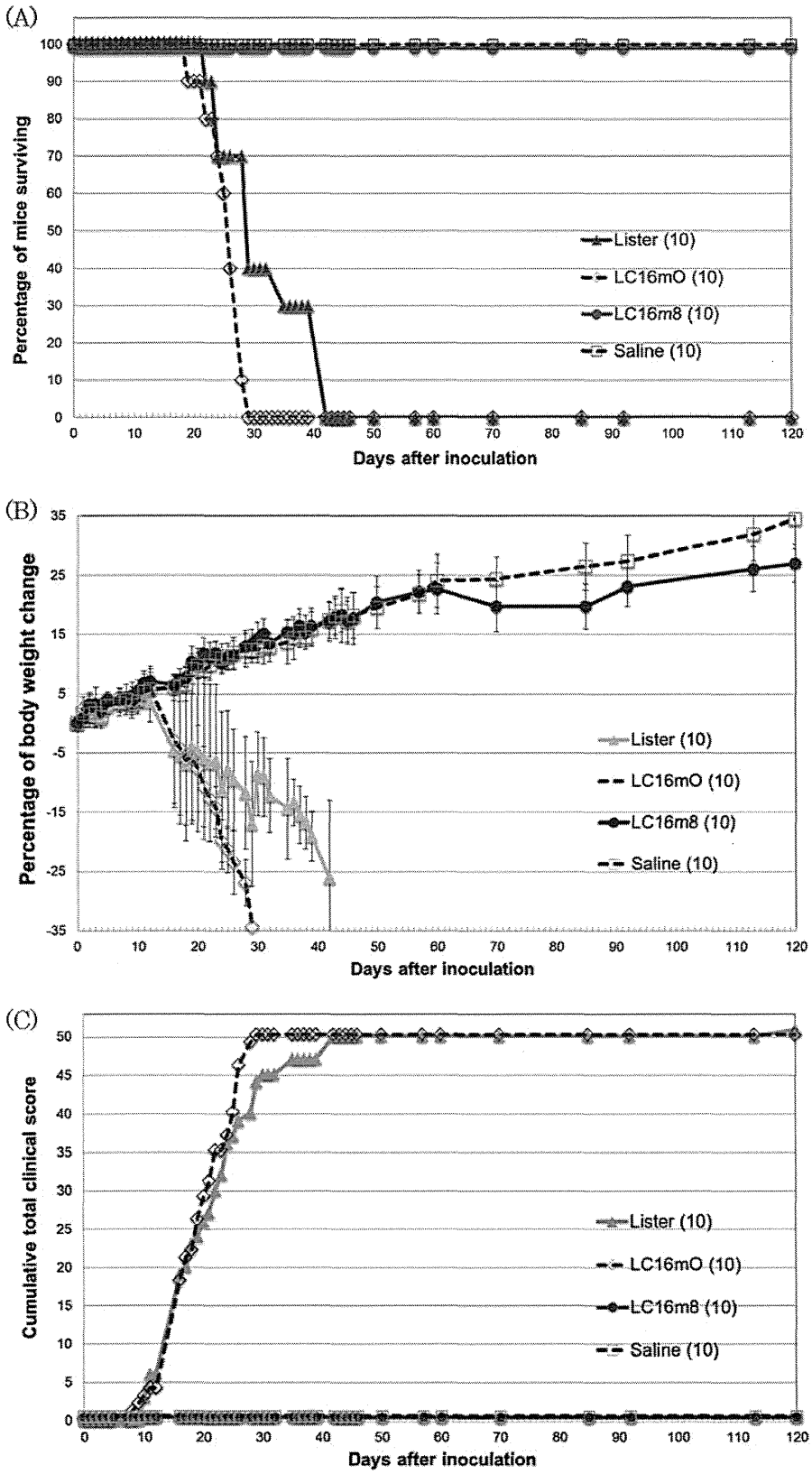

FIG 2 Effects of vaccination with Lister, LC16mO, or LC16m8 vaccine on SCID mice. Each vaccine was inoculated intraperitoneally (see details in the text). The numbers in parentheses show the numbers of animals in each experimental group. (A) Survival curve; (B) changes in body weight shown as percentages; (C) clinical score.

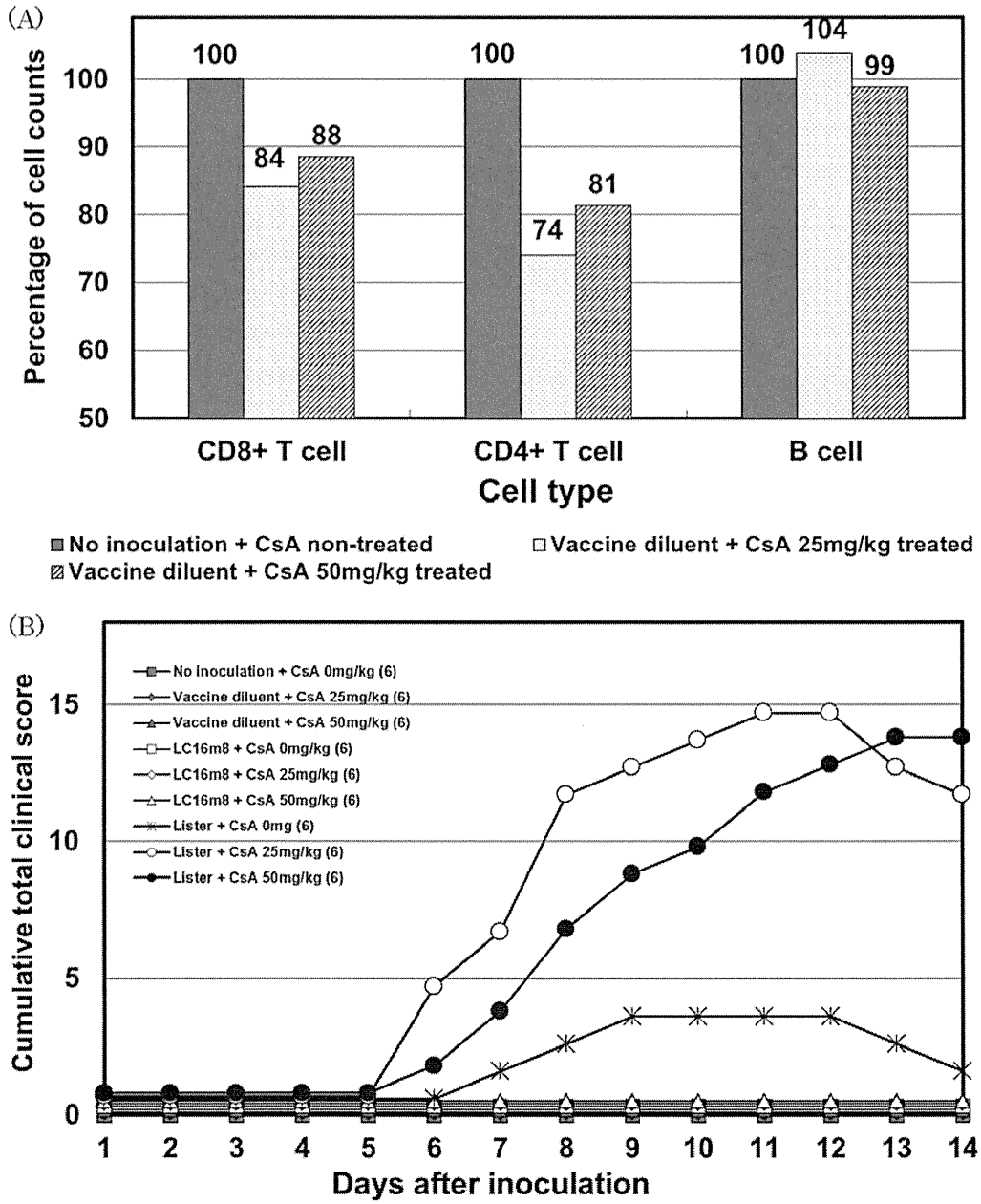

FIG 3 Effects of cyclosporine (CsA) on wild-type mice inoculated with Lister or LC16m8 vaccine. The numbers in parentheses show the numbers of animal of each experimental group. Mice were subcutaneously injected with CsA. Mice not receiving injection with CsA were used as controls (see details in the text). The numbers in parentheses show the numbers of animal of each experimental group. (A) Change in immunocyte numbers in mice after treatment with two different doses of CsA compared with those in untreated mice; in this case mice were not inoculated with any vaccine. (B) Clinical scores of the mice inoculated with LC16m8 or Lister vaccine and treated with CsA before or after vaccine inoculation.

western and central Africa where a high prevalence of HIV infection exists (12). LC16m8 protects monkeys from monkeypox (21), and because monkeypox also infects humans and causes smallpox-like disease, vaccination of subjects with LC16m8 is warranted in this region. Our studies have shown that LC16m8 vaccine may be safely used in immunodeficient subjects and that LC16m8 can also serve as a safe vector for a recombinant virus expressing foreign antigens (22).

In conclusion, we have shown that LC16m8 vaccine, recommended by the WHO SAGE as a preferred stockpile vaccine, can be safely used in suckling mice and immunodeficient mice. Our results also open the way for its use to protect against monkeypox infection in areas, such as western and central Africa, where there are many immunodeficient subjects.

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REFERENCES

1. **Henderson DA.** 2009. *Smallpox: the death of a disease.* Prometheus Books, New York, NY.
2. **Arita I.** 2010. The smallpox eradication saga: an insider's view. Orient BlackSwan, Hyderabad, India.
3. **Yamaguchi M, Kimura M, Hirayama M.** 1975. Report of the National Smallpox Vaccination Research Committee: study of side effects, complications and their treatment. Clin. Virol. **3**:269–278. (In Japanese.)
4. **Weltzin R, Liu J, Pugachev KV, Myers GA, Coughlin B, Blum PS, Nichols R, Johnson C, Cruz J, Kennedy JS, Ennis FA, Monath TP.** 2003. Clonal vaccinia virus grown in cell culture as a new smallpox vaccine. Nat. Med. **9**:1125–1130. http://dx.doi.org/10.1038/nm916.
5. **Belyakov IM, Earl P, Dzutsev A, Kuznetsov VA, Lemon M, Wyatt LS, Snyder JT, Ahlers JD, Franchini G, Moss B, Berzofsky JA.** 2003. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc. Natl. Acad. Sci. U. S. A. **100**:9458–9463. http://dx.doi.org/10.1073/pnas.1233578100.
6. **Earl PL, Americo JL, Wyatt LS, Eller LA, Whitbeck JC, Cohen GH, Eisenberg RJ, Hartmann CJ, Jackson DL, Kulesh DA, Martinez MJ, Miller DM, Mucker EM, Shamblin JD, Zwiers SH, Huggins JW, Jahrling PB, Moss B.** 2004. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature **428**:182–185. http://dx.doi.org/10.1038/nature02331.
7. **Chen RT, Lane JM.** 2003. Myocarditis: the unexpected return of smallpox vaccine adverse events. Lancet **362**:1345–1346. http://dx.doi.org/10.1016/S0140-6736(03)14674-0.
8. **Enserink M.** 2004. Biodefense. Smallpox vaccines: looking beyond the next generation. Science **304**:809. http://dx.doi.org/10.1126/science.304.5672.809a.
9. **Hashizume S, Yoshikawa H, Morita M, Suzuki K.** 1985. Properties of attenuated mutant of vaccinia virus, LC16m8, derived from Lister strain., p. pp421–428. *In* Quinnan HB (ed), Vaccinia virus as vectors for vaccine antigens. Elsevier Science Publishing Co., Amsterdam, The Netherlands.
10. **Morita M, Aoyama Y, Arita M, Amona H, Yoshizawa H, Hashizume S, Komatsu T, Tagaya I.** 1977. Comparative studies of several vaccinia virus strains by intrathalamic inoculation into cynomolgus monkeys. Arch. Virol. **53**:197–208. http://dx.doi.org/10.1007/BF01314664.
11. **World Health Organization.** 2014. Meeting of the Strategic Advisory Group of Experts on immunization, November 2013—conclusions and recommendations. Wkly. Epidemiol. Rec. **89**:1–20.
12. **Parker AK, Parker S, Yokoyama WM, Corbett JA, Buller RM.** 2007. Induction of natural killer cell responses by ectromelia virus controls infection. J. Virol. **81**:4070–4079. http://dx.doi.org/10.1128/JVI.02061-06.
13. **World Health Organization.** 2004. WHO recommendations for the production and quality control of smallpox vaccine, revised 2003. WHO Technical Report Series No. 926. World Health Organization, Geneva, Switzerland.
14. **Hiramine C, Hojo K, Matsumoto H, Koseto M, Itoh M.** 1989. Differential effect of cyclosporine in vivo on the distribution of T cell subsets in the thymus, spleen, and lymph nodes. Transplantation **47**:499–503. http://dx.doi.org/10.1097/00007890-198903000-00020.
15. **Takahashi-Nishimaki F, Funahashi S, Miki K, Hashizume S, Sugimoto M.** 1991. Regulation of plaque size and host range by a vaccinia virus gene related to complement system proteins. Virology **181**:158–164. http://dx.doi.org/10.1016/0042-6822(91)90480-Y.
16. **Smith GL, Vanderplasschen A, Law M.** 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. **83**:2915–2931.
17. **Morikawa S, Sakiyama T, Hasegawa H, Saijo M, Maeda A, Kurane I, Maeno G, Kimura J, Hirama C, Yoshida T, Asahi-Ozaki Y, Sata T, Kurata T, Kojima A.** 2005. An attenuated LC16m8 smallpox vaccine: analysis of full-genome sequence and induction of immune protection. J. Virol. **79**:11873–11891. http://dx.doi.org/10.1128/JVI.79.18.11873-11891.2005.
18. **Gordon SN, Cecchinato V, Andresen V, Heraud JM, Hryniewicz A, Parks RW, Venzon D, Chung HK, Karpova T, McNally J, Silvera P, Reimann KA, Matsui H, Kanehara T, Shinmura Y, Yokote H, Franchini G.** 2011. Smallpox vaccine safety is dependent on T cells and not B cells. J. Infect. Dis. **203**:1043–1053. http://dx.doi.org/10.1093/infdis/jiq162.
19. **Kidokoro M, Tashiro M, Shida H.** 2005. Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. Proc. Natl. Acad. Sci. U. S. A. **102**:4152–4157. http://dx.doi.org/10.1073/pnas.0406671102.
20. **von Krempelhuber A, Vollmar J, Pokorny R, Rapp P, Wulff N, Petzold B, Handley A, Mateo L, Siersbol H, Kollaritsch H, Chaplin P.** 2010. A randomized, double-blind, dose-finding phase II study to evaluate immunogenicity and safety of the third generation smallpox vaccine candidate IMVAMUNE. Vaccine **28**:1209–1216. http://dx.doi.org/10.1016/j.vaccine.2009.11.030.
21. **Saijo M, Ami Y, Suzaki Y, Nagata N, Iwata N, Hasegawa H, Ogata M, Fukushi S, Mizutani T, Sata T, Kurata T, Kurane I, Morikawa S.** 2006. LC16m8, a highly attenuated vaccinia virus vaccine lacking expression of the membrane protein B5R, protects monkeys from monkeypox. J. Virol. **80**:5179–5188. http://dx.doi.org/10.1128/JVI.02642-05.
22. **Sugimoto M, Yamanouchi K.** 1994. Characteristics of an attenuated vaccinia virus strain, LC16m0, and its recombinant virus vaccines. Vaccine **12**:675–681. http://dx.doi.org/10.1016/0264-410X(94)90215-1.